NOVEL PROGESTIN SIGNALING MOLECULES IN THE BRAIN: DISTRIBUTION, REGULATION AND MOLECULAR MECHANISM OF ACTION

A Dissertation Presented

by

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To Mom, Dad, Dan, Adrien, Jay and my closest friends, for their love and support
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ABSTRACT

NOVEL PROGESTIN SIGNALING MOLECULES IN THE BRAIN: DISTRIBUTION, REGULATION AND MOLECULAR MECHANISM OF ACTION

MAY 2011

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Progesterone regulates female reproduction in many ways, yet it is still unclear how signals are conveyed through nuclear and extranuclear receptors. The traditional notion was that progesterone binds classical progesterone receptors to alter gene transcription. This view has been challenged by the discovery of additional progesterone signaling molecules important for progesterone actions in non-neural cells. In granulosa cells, the progesterone receptor membrane component 1 (Pgrmc1) mediates progesterone effects by forming a receptor complex with binding partner, Serpine mRNA binding protein 1, but it is unknown whether these molecules function similarly in the brain. To begin to address these issues, I investigated the neural role of Pgrmc1 in female mouse brain, rat brain and in neural cells. By examining the neuroanatomical localization, hormonal regulation, and colocalization of Pgrmc1 within key neurons in the neural control of ovulation, Pgrmc1 emerged as a candidate signaling molecule likely to mediate progesterone functions. Furthermore, Pgrmc1 levels regulate the expression of several diverse genes and signaling pathways in neural cells. Taken together, these results demonstrate that Pgrmc1 function is likely to impact diverse neural functions.
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CHAPTER 1
PROGESTIN MECHANISM OF ACTION IN THE CNS

1.1 Introduction

The term “progestins” refers to a class of steroid hormones first named for a role in maintaining pregnancy (Allen and Wintersteiner, 1934). Current research shows that progestins are critical for many aspects of female reproduction and also involved in diverse non-reproductive physiological processes. In addition, emerging data suggest that progestins act in the central nervous system and affect a wide range of neural functions, such as the neural control of ovulation, sexual behaviors and neuroprotection. Despite the prominent role of progestins and the long history of research on this hormone, the mechanisms that underlie progestin cellular actions remain an area of active research. This chapter will review progestin mechanisms of action and focus on signaling molecules that may mediate the neural effects of progestin in females.

1.2 Sources and Synthesis

Progestins vary widely in their chemical structures, and may be classified into natural and synthetic types. The natural progestin, progesterone ($P_4$), is a hormone produced in the gonads, adrenal glands, and central nervous system. $P_4$ is an intermediate in the production of mineralocorticoids, glucocorticoids, androgens and estrogens. The key reactions in $P_4$ biosynthesis can be viewed in Figure 1. Like other steroid hormones, the synthesis of $P_4$ begins with cholesterol as a substrate. Cholesterol is transported from the outer to the inner mitochondrial membrane by the steroid acute regulatory protein (StAR) (Clark et al., 1994). StAR is activated by a phosphorylation event and this is regarded as the rate-limiting step in steroidogenesis. Once transported
to the inner mitochondrial membrane, a P450 side chain cleavage enzyme (P450scc) converts cholesterol to pregnenolone by removal of the C-27 cholesterol side chain (Juengel et al., 1995, Niswender, 2002). Through the actions of 3β-hydroxysteroid dehydrogenase (3β-HSD), pregnenolone is converted to P₄. While diverse cells are capable of P₄ production, the stimulatory factors that drive this process are tissue-specific.

### 1.2.3 The Nervous System

The idea of neurosteroid production has been supported by studies for over 50 years. Some of the early studies on this topic showed that the central nervous systems contains factors necessary for steroidogenesis including cholesterol sulfate (Iwamori et al., 1976), aromatase (Denef et al., 1973), steroid-5α-reductase (Jaffe, 1969, Sholiton and Werk, 1969), steroid hydroxylases (Guiraud et al., 1979, Fishman et al., 1980), hydroxysteroid dehydrogenase and steroid dehydroxylases (Knapstein et al., 1968). Another early study showed that pregnenolone levels remain elevated above plasma levels in the rat brain, even after gonadectomy and adrenalectomy (Corpechot et al., 1982).
1981, Corpechot et al., 1983). Thus, early data suggested that progesterone is produced within the nervous system.

More recent evidence supports this concept. For example, P₄ can be produced by oligodendrocytes and neurons, though astrocytes are the main steroidogenic cells of the brain (Zwain and Yen, 1999). These brain cells contain enzymes required for P₄ synthesis (Karri et al., 2007), many of which are sensitive to 17β-estradiol (E₂). E₂ increases 3β-HSD expression and activity in female rat hypothalamus (Micevych et al., 2003). In astrocytes, E₂ activates protein kinases that mediate StAR phosphorylation, resulting in P₄ production (Sinchak et al., 2003, Boulware et al., 2005, Micevych et al., 2007). Although the stimuli for P₄ synthesis vary by tissue, most steroidogenic pathways require activation of phosphorylation cascades that activate StAR and other steroidogenic enzymes.

In the peripheral nervous system, similar mechanisms enable neurons and Schwann cells to produce P₄. Schwann cells possess the enzymes necessary for synthesis of P₄ (Guennoun et al., 1997), and produce this steroid hormone following neuronally-derived cues (Robert et al., 2001). These cues are not yet known, but neural input to Schwann cells is necessary for P₄ synthesis and maintain myelination (Chan et al., 1998b). Following acute trauma, such as axonal injury, Schwann cells are stimulated to increase P₄ production (Koenig et al., 1995). In turn, the P₄ provides neuroprotective benefits including decreased cell death and increased myelin repair (Labombarda et al., 2006). Thus, P₄ biosynthesis in the nervous system may provide important neuroprotective effects that aid in myelin formation.
1.3 Effects of P₄ in the Central Nervous System

Although the effects of P₄ in the brain are diverse, much of the research focuses on two main topics: the important role of P₄ in neuroprotection and in female reproduction. The therapeutic potential of understanding P₄ actions is highlighted by studies involving brain injury and age-related disease. Additionally, the role of P₄ in the neural control of ovulation and feminine sex behaviors have been intensely studied in the field of neuroendocrinology. A summary of these studies is provided in the following sections.

1.3.1 Neuroprotection

Though the exact mechanisms are not yet clear, the findings of many studies support the idea the therapeutic P₄ administration following traumatic brain injury, stroke or spinal cord trauma improves recovery. For example, P₄ downregulates inflammatory cascades and decreases proapoptotic gene expression when administered following traumatic brain injury (He et al., 2004, Pettus et al., 2005, Guo et al., 2006, O'Connor et al., 2007). Additionally, P₄ reduces edema through a protective and reparative effect on the blood brain barrier, a structure with cells especially vulnerable to damage (Wahl et al., 1993). Damage to this structure increases edema, and P₄ exerts protective effects by inhibiting ion transport and reducing free radical-induced damage of the blood brain barrier cells (Betz and Coester, 1990a, b, Hoffman et al., 1996). Thus, P₄ may benefit and improve several components necessary for brain healing following trauma.

Many mechanisms of P₄ action have been proposed based on observations that P₄ treatment limits tissue damage and improves functional outcomes. Early on, studies
showed that $P_4$ metabolites activate $GABA_A$ receptors, thereby reducing excessive excitotoxicity (Kokate et al., 1994). The beneficial role of $P_4$ in recovery is likely to involve the classical $P_4$ receptor as demonstrated by studies using transgenic animals (Jodhka et al., 2009). Although continuing work focuses on these mechanisms, the use of $P_4$ as a therapeutic strategy has already been demonstrated in many animal studies (Gibson et al., 2008). Such findings led to clinical research in human patients and showed $P_4$ administration in victims of traumatic brain injury lowers mortality and may decrease disability levels (Wright et al., 2007). Further clinical studies are underway and research continues to reveal the mechanisms through which $P_4$ benefits neuronal survival.

1.3.2 Reproductive Functions

While the functions of $P_4$ are diverse, this hormone is primarily known for its role in female reproduction. Many $P_4$ effects, including the generation of luteinizing hormone (LH) surge that precedes ovulation, require the contribution of $E_2$. Ovarian follicles are stimulated to grow by follicle stimulating hormone each cycle, producing increasing levels of $E_2$ that feed back onto cells of the hypothalamus and anterior pituitary. $E_2$ positive feedback results in peak $E_2$ levels that increase the firing rate of gonadotropin releasing hormone (GnRH) neurons (Christian et al., 2005). This process is likely to involve the preoptic area (POA), a region that contains the anteroventral periventricular nucleus (AVPV). Ablation studies (Wiegand et al., 1980, Ronnekleiv and Kelly, 1986) or estrogen deprivation (Petersen et al., 1989) in the AVPV blocks LH surge, and neurons of the AVPV contain most of the $E_2$ receptors in the POA and provide $E_2$-sensitive input to GnRH neurons (Simonian et al., 1999, Ottem et al., 2004).
In the AVPV, E₂ induces expression of the classical P₄ receptor (Pgr), (Simerly et al., 1996, Hagihara et al., 1992, Shughrue et al., 1997), an induction required for the GnRH hyperactivation, LH surge release (Chappell and Levine, 2000) and ovulation. The ruptured follicle that remains forms a corpus luteum that produces additional P₄. While circulating P₄ activates the Pgr, the ligand-independent activation of the Pgr by neural signals is thought to be also critical for the LH surge mechanism (Mani et al., 1994a, Mani et al., 1996). Thus, the E₂-dependent Pgr induction and possibly ligand-independent activation of PR are crucial for the preovulatory LH surge.

While studies in ovariectomized rats show that E₂ treatment alone is sufficient for a blunted daily LH surge, P₄ treatment administration to E₂-primed rats rapidly advances the onset of ovulation, and increases the magnitude and duration of the LH surge (Everett, 1948, Rothchild, 1965, DePaolo and Barraclough, 1979). Interestingly, intact animals do not exhibit significantly increased circulating P₄ levels preceding the LH surge (Smith et al., 1975, Park and Ramirez, 1987). Pgr activation is required for LH surge initiation, and may occur by ligand-independent manner through neural activity (Chappell et al., 2000). Another recent possibility is that hypothalamic P₄ production activates E₂-induced Pgrs in the intact animal. This idea is supported by the finding that LH surge levels are correlated with hypothalamic P₄ concentrations, an observation that persists in animals that lack ovarian and adrenal sources of P₄ (Micevych et al., 2003, Micevych and Sinchak, 2008). Finally, the LH surge can be blocked by hypothalamic administration of P₄ synthesis inhibitors (Snyder et al., 1984, DePaolo, 1988, Hibbert et al., 1996). Together these data suggest that both the E₂-induction of Pgr and neuroprogesterone synthesis may be requisite steps in the LH
surge mechanism. Though many studies have investigated the role of P₄ in this process, the possibility that P₄ signaling molecules may mediate the rapid advancement and amplification of the surge remains untested.

Beyond a role in the neural control of ovulation, P₄ is also implicated in the coordinated facilitation of reproductive sex behaviors that accompany ovulation. Following E₂-priming, P₄ activation of Pgr within the ventromedial nucleus of the hypothalamus (VMN) induces lordosis, a stereotypical receptive posture (Whalen, 1974, Moguilewsky and Raynaud, 1979, Pfaff and Sakuma, 1979, Hoshina et al., 1994). Similar to the LH surge mechanism, this P₄ effect requires to E₂-induction of the Pgr, underscoring the important transcriptional role of E₂ (Parsons et al., 1980, Pollio et al., 1993, Mani et al., 1994b, Ogawa et al., 1994). Interestingly, membrane-active P₄ metabolites and membrane impermeable P₄-BSA are also capable of facilitating receptive behaviors in E₂-primed rats (Rodriguez-Manzo et al., 1986, Ke and Ramirez, 1987), suggesting that additional mechanisms of P₄ actions synchronize ovulation and the expression of feminine sex behaviors. Rapid membrane-initiated P₄ signaling may be mediated through progestin signaling molecules, though this possibility has not yet been explored. While clues as to these behaviors continue to emerge, it is clear that Pgr induction is a critical component in the control of sex behavior.

1.4 The Classical Progesterone Receptor (Pgr)

Studies using Pgr knockout mice show that the classical Pgr mediates many P₄ effects in reproductive tissues (Ismail et al., 2003). The Pgr gene is expressed as two protein isoforms, A and B, that have identical ligand- and DNA-binding domains. These isoforms only differ in that Pgr B has an additional 164 amino acids at
the C-terminus (Kastner et al., 1990). Results of studies in Pgr knockouts predict that each splice variant has distinct reproductive roles. For example, Pgr A knockouts are characterized by ovary and implantation defects, while Pgr B knockouts show proliferative defects in the mammary gland and uterus (Shyamala et al., 1998, Mulac-Jericevic et al., 2000, Mulac-Jericevic et al., 2003). The ratio of A and B isoforms also appears to be important for normal growth and development, and disruption of this ratio is a common feature of human breast cancers (Mote et al., 2004). Though these studies address the roles of specific Pgr isoforms, the interpretation of such findings is complicated due to developmental differences and compensation inherent to body-wide gene targeting approaches.

Though Pgr is a member of the nuclear receptor superfamily of transcription factors (Evans, 1988, Tsai et al., 1988), not all Pgr-dependent P₄ effects include delayed-onset transcriptional events. Short latency Pgr-dependent effects have also been observed (Meyerson, 1972, Kubli-Garfias and Whalen, 1977, Schumacher et al., 1990), and they do not require Pgr DNA-binding activity. Specific examples include findings that P₄ binds Pgrs to activate phospholipase C and kinase Cdk1 in enucleated amphibian oocytes (Schuetz, 1977, Bayaa et al., 2000, Morrison et al., 2000). In mammalian cell lines, P₄ causes Pgr-dependent activation of p60-Src kinase within minutes (Migliaccio et al., 1998), and also rapidly and transiently activates MAPK signaling (Boonyaratanakornkit et al., 2001). Such findings prompted revision of the traditional tenet of steroid hormone action (Truss and Beato, 1993), as the mechanism of Pgr action extends beyond gene transcription.
Efforts to understand the extranuclear actions of Pgr have shown that polyproline motifs of the Pgr enable interactions with Src, a tyrosine kinase (Boonyaratanakornkit et al., 2001). This interaction between Pgr and Src does not require Pgr-DNA binding nor transcription, suggesting that the Pgr plays dual roles in cell signaling and transcription that are distinct and separable (Edwards et al., 2002). Although both Pgr isoforms have identical Src-interacting polyproline motifs, the A isoform is predominantly located in the nucleus and is not capable of activating Src (Boonyaratanakornkit et al., 2007). Thus, it is likely that the ratio of Pgr isoforms, along with tissue-specific factors, contribute to nuclear and extra-nuclear P_4 actions in the brain and reproductive tissues. Importantly, additional P_4 signaling molecules may impact Pgr function. These findings underscore the complexity of Pgr-dependent P_4 signaling and suggest that multiple signaling mechanisms may mediate diverse P_4 actions.

1.5 Progesterone Receptor Membrane Component-1 (Pgrmc1)

Pgrmc1 is a progestin signaling molecule that was discovered in several unrelated biological contexts, resulting in interesting clues as to physiological functions, but also many synonyms (Losel et al., 2003, Min et al., 2004, Cahill, 2007). Pgrmc1 encodes a 28-kDa protein that is often isolated as a 56-kDa dimer, and contains a single N-terminal membrane-spanning domain, a cytochrome b5-like or steroid/heme binding domain (Mifsud and Bateman, 2002). This protein has been localized to the plasma membrane (Peluso et al., 2006), endoplasmic reticulum (ER) (Falkenstein et al., 1998), and nucleus (Beausoleil et al., 2004). Pgrmc1 has been implicated in a wide array of physiological processes, leading to the publication of many reviews despite limited
understanding of its exact cellular roles (Craven, 2008, Guennoun et al., 2008, Sakamoto et al., 2008, Rohe et al., 2009).

Among the first identified Pgrmc1 functions is a role in steroidogenesis, an idea supported by studies in several cell types that demonstrate associations with endoplasmic reticulum (ER) proteins (Laird et al., 1988, Nolte et al., 2000). In adrenal gland cells, Pgrmc1 may be required for activities of CYP21 and CYP11B1 (Min et al., 2004), two cytochrome P450 enzymes that catalyze the 21-hydroxylation of P₄ and 18-hydroxylation of deoxycorticosterone, respectively (Barker et al., 1992). In yeast, the Pgrmc1 homologue binds and activates CYP51A1, an enzyme that controls the rate of cholesterol synthesis (Hughes et al., 2007). Additionally, the same authors showed that Pgrmc1 activates Erg5/CYP61A1, another P450 protein in the cholesterol synthesis pathway. In COS7 kidney cells, Pgrmc1 binds to insulin-induced gene and SREBP cleavage activating protein, two endoplasmic reticulum proteins critical for cholesterol homeostasis (Suchanek et al., 2005, Goldstein et al., 2006). Additional evidence that supports a role in cholesterol or steroid metabolism is that Pgrmc1 is found in cells of the zona fasciculata/reticularis (adrenal gland), Leydig cells and hepatocytes (Raza et al., 2001).

The brain is another location found to express Pgrmc1, and several studies have identified potential roles for Pgrmc1 in steroidogenesis within particular neurons. Cerebellar Purkinje cells contain P450<sup>sec</sup>, 3β-HSD and StAR, and produce P₄ during development that is correlated with Pgrmc1 expression (Ukena et al., 1998, Ukena et al., 1999, Sakamoto et al., 2004). High levels of Pgrmc1 expression were detected in the ER of these neurons during times of elevated P₄ production (Sakamoto et al., 2008).
P₄ biosynthesis from Purkinje neurons is required for dendritic growth and synaptogenesis (Sakamoto et al., 2001), indicating that Pgrmc1 may be required for P₄-induced developmental processes.

Pgrmc1 expression in development is also elevated along the floor plate and notochord of developing murine brain, suggesting a role in axon guidance (Runko et al., 1999). Indeed, one of Pgrmc1’s early synonyms is ventral midline antigen (VEMA). Pgrmc1 expression shares a very close distribution pattern to molecules required for axon guidance at the midline in the developing spinal cord and optic chiasm (Ho et al., 1994, Runko and Kaprielian, 2002). More support for a role in development is provided by work on the C. Elegans ortholog of Pgrmc1, vem-1. Animals treated with vem-1 siRNA and vem-1-null mutants are characterized by aberrant neuronal projections and midline neurons that fail to reach their ventral positions (Runko and Kaprielian, 2004). Taken together, these findings on Pgrmc1’s role in axon guidance provide intriguing clues, but unequivocal evidence for this idea is lacking as research on this topic is very limited.

In the adult rat brain, very little is known regarding the function of Pgrmc1. Localization studies lead to proposed roles of Pgrmc1 in water homeostasis and neuroendocrine functions (Krebs et al., 2000, Meffre et al., 2005). The actual cellular role of Pgrmc1 is much less clear, but one possibility is a role in P₄ signaling, as suggested by findings in ovarian cells. For example, Pgrmc1 mediates the anti-apoptotic effects of P₄ in granulosa cells (Peluso et al., 2001). These rapid P₄ actions require that Pgrmc1 interact with a binding partner, Serpine1 mRNA binding protein 1 (Peluso et al., 2005), but whether this interaction persists in neural cells is not known.
The current state of research on Pgrmc1’s role in the brain poses many questions. Despite multiple links between P₄ and Pgrmc1, it is not known if Pgrmc1 is required to mediate P₄ actions in the brain. Furthermore, the neuroanatomical localization of Pgrmc1 in the brain is largely unknown, and the factors that induce Pgrmc1 expression have not yet been identified. The expression of Pgrmc1 has only been reported in vasopressinergic (Meffre et al., 2005) and Purkinje cells (Sakamoto et al., 2004), neural phenotypes confined to distinct brain regions. To begin to address these questions, the following chapters report my research conducted to test the hypothesis that Pgrmc1 has important functions in the brain.
1.6 References


Hoshina Y, Takeo T, Nakano K, Sato T, Sakuma Y (1994) Axon-sparing lesion of the preoptic area enhances receptivity and diminishes proceptivity among


CHAPTER 2
DISTRIBUTION OF mRNAs ENCODING CLASSICAL PROGESTIN RECEPTOR, PROGESTERONE MEMBRANE COMPONENTS 1 AND 2, SERPINE mRNA BINDING PROTEIN 1, AND PROGESTIN AND ADIPOQ RECEPTOR FAMILY MEMBERS 7 AND 8 IN RAT FOREBRAIN

2.1 Abstract

Several lines of evidence suggest the existence of multiple progestin receptors that may account for rapid and delayed effects of progesterone in the central nervous system. The delayed effects have been long attributed to activation of the classical progestin receptor (Pgr). Recent studies have discovered novel progestin signaling molecules that may be responsible for rapid effects, including progesterone receptor membrane component 1 (Pgrmc1), Pgrmc2, progestin and adipoQ receptor 7 (Paqr7) and Paqr8. The functions of these molecules have been investigated extensively in non-neural, but not in neural tissues, partly because it is unclear which are expressed in the brain and where they are expressed. To address these issues, we compared the distributions of mRNAs encoding Pgr, Pgrmc1, Pgrmc2, Paqr7 and Paqr8 using in situ hybridization (ISH) with radiolabeled oligodeoxynucleotidyl probes in forebrain tissues of estradiol-treated female rats. We also examined the distribution of serpine mRNA binding protein 1 (Serbp1), a putative binding partner of Pgrmc1. Analysis of adjacent brain sections showed that the highest expression of mRNAs encoding Pgr, Pgrmc1, Pgrmc2 and Serbp1 was detected in several hypothalamic nuclei important for female reproduction. In contrast, expression patterns of Paqr7 and Paqr8 were low and
homogeneous in the hypothalamus, and more abundant in thalamic nuclei. The neuroanatomical distributions of these putative progestin signaling molecules suggest that Pgrmc1 and Pgrmc2 may play a role in neuroendocrine functions while Paqr7 and Paqr8 are more likely to regulate sensory and cognitive functions.

2.2 Introduction

Progesterone (P₄) is widely recognized for its ability to regulate neural functions related to reproduction, but it also affects diverse processes such as cognition and neurogenesis (Berman et al., 1997, Giachino et al., 2003). The traditional tenet of P₄ action is that it binds the cognate progestin receptor (Pgr), and functions as a ligand-activated transcription factor to regulate gene expression. However, rapid non-genomic effects have also been reported (Meyerson, 1972, Parsons et al., 1980, Mani et al., 1994b), and P₄ can act in the absence of Pgr (Frye et al., 2006). These data support the emerging concept that P₄ actions in the brain may be through the classical Pgr and also through non-classical mechanisms.

Current research suggests that there are several possible candidates for mediating the non-classical effects of P₄. One such protein is progesterone receptor membrane component 1 (Pgrmc1), but it does not appear to function as a traditional receptor because it requires a binding partner known as serpine mRNA binding protein 1 (Serbp1) (Peluso et al., 2005, 2006). Moreover, the structure of Pgrmc1 does not share homology with either classical steroid receptors or G-coupled protein receptors (Mifsud and Bateman, 2002). Nonetheless, Pgrmc1 mediates several important Pgr-independent effects. For example, P₄ acts through Pgrmc1 to activate phosphoinositide-dependent protein kinase 1 and phosphorylate Akt (Hand and Craven, 2003). In the
ovary. Pgrmc1 and Serbp1 form a receptor complex required for the antiapoptotic effects of P₄ in granulosa cells (Peluso et al., 2006, Zhang et al., 2008). Pgrmc2 is a closely related isoform of Pgrmc1, differing mainly in its N-terminus, but there is virtually no information regarding Pgrmc2 function (Falkenstein et al., 1999, Peluso et al., 2005). Pgrmc1 has been localized to several brain regions (Krebs et al., 2000, Sakamoto et al., 2004, Meffre et al., 2005), but no studies have systematically mapped its distribution and the role of this protein in the brain remains unknown. Likewise, no studies have mapped neural expression of Serbp1 or Pgrmc2. Despite these limitations, several lines of evidence indicate that Pgrmc1/Serbp1, and possibly Pgrmc2, may be important for non-classical P₄ actions in the brain.

Two other candidates for mediating the non-genomic effects of P₄ are progestin and adipoQ receptor 7 (Paqr7) and Paqr8. These are G-protein coupled receptors first discovered in spotted sea trout, and subsequently in mammalian tissues (Zhu et al., 2003a, Zhu et al., 2003b). Although activation of these receptors by P₄ regulates cAMP levels and MAPK activity in fish (Hanna et al., 2006), there is some debate about whether they function as bona fide P₄ receptors in mammals (Fernandes et al., 2008). Recent reports detected Paqr7 and Paqr8 mRNAs in hypothalamic tissue of mice, but the exact anatomical localization is unknown (Sleiter et al., 2009). Collectively, these findings raise the possibility that Paqr7 and Paqr8 also mediate P₄ neural actions.

Although there is abundant evidence that these signaling molecules participate in P₄ signaling, it is unclear which are important in the nervous system. Moreover, while many neural functions are modulated by P₄, there is little information about which functions require Pgr, non-nuclear receptors or both. One obstacle to resolving this
question is that neither the classical Pgr nor any of the non-classical P₄ receptor candidates have been systematically mapped in the brain. To address this issue, we used in situ hybridization histochemistry (ISHH) to map the expression of mRNAs encoding Pgr, Pgrmc1, Pgrmc2, Serbp1, Paqr7 and Paqr8. In these studies, we used female rats because of the important role P₄ plays in regulation of female-specific physiological functions.

2.3 Materials and Methods

2.3.1 Animals and Tissue Preparation

All protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts and all animals were housed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Six adult female Sprague-Dawley rats (225-250 g; approximately 95 days of age; Harlan Sprague-Dawley, Madison WI) were individually housed in the Animal Care Facility on a 14:10 light:dark cycle with food and water provided ad libitum. To achieve a similar hormonal milieu among rats, we ovariectomized them and implanted two Silastic capsules containing E₂ (150 µg/ml 17β-estradiol in sesame oil) a week later as described previously (Petersen and LaFlamme, 1997). Twenty-four hours later, we collected brains and rapidly froze and stored them at -80 °C until they were cryosectioned (Leica CM3000, Nussloch, Germany).

For three animals, 14-µm coronal forebrain sections were obtained and thaw-mounted onto gelatin-coated slides and stored at -80 °C until ISHH was performed. The remaining three animals were used for RNA isolation in validation studies described below.
2.3.2 Probe Preparation

In these studies, we used oligodeoxynucleotidyl probes of the same length and specific activity. Antisense oligodeoxynucleotide sequences used for end-tailing are provided in Table 2.6.1. Both sense and antisense sequences were produced by an automated DNA synthesizer and purified by reverse-phase HPLC by Integrated DNA Technologies (Coralville, IA). Oligodeoxynucleotides were 3’-end labeled with [α\(^{33}\)P]-dATP (PerkinElmer, Waltham, MA) using terminal deoxynucleotidyl transferase (Roche, Indianapolis, IN) as described previously (Petersen et al., 1989). Incubation was halted by addition of TE (10mM Tris-HCL; pH 8.0, 1 mM EDTA), and the probe was purified by phenol-chloroform extraction and ethanol precipitation. The resulting pellet was washed with 70% ethanol and resuspended in 25 µl TE.

2.3.3 ISHH

The distribution pattern for each mRNA was determined in separate ISHH runs, and tissue sections were prehybridized as previously described (Ottem et al., 2004). Radioisotopic probes (0.5 x 10\(^6\) cpm) were applied directly to brain tissue in 20 µl hybridization buffer. This buffer contained 4XSSC (1XSSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.2), 50% (v/v) formamide, 10% (w/v) dextran sulfate, 250 µg/ml yeast tRNA, 1X Denhardt's solution, 500 µg/ml heparin sodium salt, 0.1% sodium pyrophosphate and 0.05 M dithiothreitol added freshly before use. Sections were covered with glass coverslips and hybridized overnight at 37 °C in humidified plastic boxes. Slides were removed from 37 °C and allowed to cool, and coverslips were floated off in 1XSSC. They were washed four times for 15 minutes each in 2XSSC-50% formamide solution at 40 °C, followed by four washes, 15 minutes each, in
1XSSC. Finally, slides were rinsed in water and briefly dehydrated in 70% ethanol. The slides were air-dried and apposed to Kodak BioMax MR film (Rochester, NY) for signal detection. In order to acquire optimum signal, autoradiograms were developed at 1, 3 and 6 weeks by an X-ray film processor and images were acquired using BioQuant Imaging Software (Bio-Quant Inc, Nashville, TN,) and a CCD videocamera (QImaging QICAM FAST color).

2.3.4 Validation of probe specificity

To determine the specificity of the hybridization signal, sense strand probes to each target of interest were hybridized to representative sections. To verify specificity of each antisense probe, subsets of adjacent slides were treated with RNase A solution (100 µg/ml RNase A in 0.5 M NaCl, 0.05 M EDTA and 0.01 M Tris-HCl) for one hour at 37 °C following prehybridization. An additional set of slides was used for Nissl staining in order to provide reference material for identification of specific brain regions.

Regardless of exposure time, Paqr7 and Paqr8 antisense probes produced diffuse and homogeneous signal, therefore multiple probes (Table 2.6.2) were used for each gene to verify signal specificity. To ensure specificity in regions that displayed low ISHH signal for Paqr7, quantitative polymerase chain reaction (QPCR) was performed using cDNA derived from RNA of the diagonal band of Broca and striatum, regions with two different signal intensities. RNA was isolated from tissue punches using the RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA) and reverse transcribed into cDNA using Quantitect Reverse Transcription Kit (Qiagen) and manufacturer’s protocol. Reactions were performed in a Stratagene Mx3000P instrument programmed as
follows: 95 °C, 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec.

Reactions contained reagents from QuantiTect SYBR Green Kit and manufacturer’s protocols were used (Roche Diagnostics, Indianapolis, IN). Specific primer sets were obtained from Integrated DNA Technologies, and the forward and reverse primers used to detect Paqr7 mRNA were 5’-TGCACCGCATCATAGTGC-3’ and 5’-GATAGTCCAGCGTCACAGC-3’. Resulting cycle thresholds were normalized using forward and reverse primers for beta-actin: 5’-GGGAAATCGTGCCTGACATT-3’ and 5’- GCGGCAGTGGCCATCTC-3’. Samples with no cDNA were used as negative controls. Products were resolved using 2% agarose gel electrophoresis.

2.3.5 Data Analysis

Neuroanatomic mapping of Pgrmc1, Pgrmc2, Paqr7, Paqr8, Serbp1 and Pgr was performed with the aid of a rat brain atlas (Swanson, 1998) and Nissl-stained adjacent sections. Relative levels of mRNA were determined by optical densitometric measurements of the autoradiographic signals across different brain regions. Results of this semiquantitative ISHH were obtained by digitizing all autoradiographic images and four ranges of density of labeling were used to determine relative intensities across brain regions and across probes. Signal strength intensity was determined using arbitrary optical density units and denoted by – (background; 0-51), + (low; 52-102), ++ (moderate; 103-153), +++ (154-204), and ++++ (highest signal intensity; 205-255). Digitized images were imported into Adobe Photoshop 8.0 CS (Adobe Systems Inc., San Jose CA) and all figures were cropped to the same size for display.

For QPCR studies, relative levels of Paqr7 mRNA were analyzed using the 2-ΔΔCt method (Livak and Schmittgen, 2001).
2.4 Results

2.4.1 Probe Specificity

We verified the specificity of the probes used in these studies in several ways. All four oligodeoxynucleotidyl probes for each target produced the same labeling pattern. In addition, neither sense strand controls nor RNAse A-treated sections showed specific signal with the exception of the sense probe for Paqr7. Although Paqr7 sense strand control probes showed signal above background, the signal for antisense probes was abolished by tissue pretreatment with RNAse A (Fig. 2.7.1, D3). Moreover, QPCR detected a single band of expected size using primers to Paqr7. Consistent with our ISHH findings, QPCR detected higher mRNA levels in the diagonal band of Broca than in the striatum (data not shown).

2.4.2 Distribution of Pgrmc1, Serbp1, Pgrmc2, Paqr7, Paqr8 and Pgr mRNAs

The neuroanatomical distributions of mRNAs encoding Pgrmc1, Serbp1, Pgrmc2, Paqr7, Paqr8 and Pgr are presented in Table 2.6.3. The distributions of mRNAs encoding Pgrmc1, Pgrmc2 and Serbp1 overlapped extensively throughout the brain, and some of these regions displayed high levels of the classical Pgr, most notably in ventral structures. Paqr7 and Paqr8 mRNAs were not highly expressed in these structures, but showed expression patterns similar to one another. Unlike signals for probes to Pgrmc1, Pgrmc2, Serbp1 and Pgr, signals for Paqr7 and Paqr8 probes were characterized by diffuse labeling found predominantly in the thalamus.

2.4.3 Diencephalon

In the rostral diencephalon, high signal intensities for Pgrmc1 mRNA were detected in several olfactory nuclei as indicated in Fig. 2.7.1, A1. We found moderate
signal for mRNAs encoding Pgrmc1, Paqr7 and Paqr8 in the diagonal band of Broca (Fig. 2.7.1; A1, D1 and E1, respectively). In the preoptic area (POA), the AVPV nucleus displayed very high levels of mRNAs encoding Pgrmc1 and Pgr (Fig. 2.7.2, A1 and F1). Serbp1 and Pgrmc2 also showed elevated signal intensities in this nucleus (Fig. 2.7.2, B1 and C1). Although Paqr7 and Paqr8 mRNAs were found in the POA, clearly defined signal within the AVPV was not apparent (Fig. 2.7.2, D1 and E3). The bed nucleus of the stria terminalis contained dense labeling for Pgrmc1, Serbp1 and Pgr (Fig. 2.7.2, A2, B2 and F2). Moderate levels of mRNAs encoding Pgrmc1 and Paqr8 were found in the rostral portion of the lateral septal nucleus (Fig. 2.7.2, A1 and E1). The paraventricular nucleus (PVN) and supraoptic nucleus (SON) showed abundant labeling for Pgrmc1, Serbp1, and Pgrmc2 (Fig. 2.7.2, A3, B3 and C3). High signal for probes encoding these mRNAs was also detected in the zona incerta, ventromedial nucleus (VMH) and arcuate nucleus (Arc), a finding consistent with the distribution of Pgr (Fig. 2.7.3, A1, B1, C1 and F1). The reunions nucleus of the thalamus showed moderate levels of mRNAs encoding Pgrmc1, Serbp1, Pgrmc2 and Pgr mRNAs (Fig. 2.7.3, A1, B1, C1 and F1). In contrast, probes for these mRNAs showed low levels of signal intensity in the ventromedial thalamic nuclei while those encoding Paqr7 and Paqr8 were somewhat prominent (Fig. 2.7.3, column 1). The anterodorsal and anteroventral thalamic nuclei displayed similarly elevated levels of Serbp1 and Paqr8 gene expression (Fig. 2.7.3, B1 and E1).

**2.4.4 Telencephalon**

The hippocampus showed intense hybridization signals for Pgrmc1, Serbp1, Pgrmc2 and Pgr probes as shown in Fig. 2.7.3, columns 1 and 2. Within this region,
signal intensities were highest in the CA2 and CA3 for all four mRNAs. In the dentate
gyrus, stronger signal was detected for mRNAs encoding Pgrmc1, Serbp1 and Pgrmc2
(Fig. 2.7.3, A2, B2 and C2) than for those encoding Pgr (Fig. 2.7.3, F2). Paqr8
displayed low to moderate signal intensity within the hippocampus (Fig. 2.7.3, E2). In
contrast, mRNAs encoding Paqr7 were undetectable in CA1, CA2, CA3 and dentate
gyrus (Fig. 2.7.3, D1 and D2).

In the cortex, moderate levels of mRNAs were observed for Pgrmc1, Serbp1,
Pgrmc2, Paqr8 and Pgr as shown in column 2 of Fig. 2.7.1. High signal intensity was
detected in cortical regions for Pgrmc1, Serbp1 and Pgr mRNA (Fig. 2.7.3, row A, B
and F). The medial amygdala showed moderate expression of Pgrmc1, Serbp1, Pgrmc2
and Pgr (Fig. 2.7.3, columns 1 and 2). Alternatively, Paqr7 and Paqr8 signals in the
amygdala were low overall (Fig. 2.7.3, D2 and E2).

The highest levels of mRNAs encoding Pgrmc1, Serbp1 and Pgr in the caudal
forebrain were detected in the periaqueductal gray (PAG) (Fig. 2.7.3, A3, B3 and F3).
Pgrmc2 mRNA levels were moderately high within this region (Fig. 2.7.3, C3), but
Paqr7 and Paqr8 mRNAs were very low (Fig. 2.7.3, D3 and E3). Interestingly, Paqr8
mRNA levels were highest in the nucleus of the oculomotor tract (Fig. 2.7.3, E3).
Finally, within the substantia nigra and interpeduncular nucleus, we detected
moderately high levels of mRNAs encoding Pgrmc1, Serbp1 and Pgr (Fig. 2.7.3, A3,
B3 and F3).

2.5 Discussion

Collectively, these neuroanatomical mapping data provide important clues as to
functions of putative progestin signaling molecules. The high levels of Pgrmc1 and
Pgrmc2 mRNAs within virtually all hypothalamic nuclei suggest that these molecules are likely candidates for mediating rapid neuroendocrine effects. Moreover, signals for Pgrmc1 and Pgrmc2 mRNAs were strongest in regions that also contained Pgr and regulate reproductive functions, consistent with the idea that these functions require both rapid and delayed actions of P₄. Pgrmc1 and Serbp1 mRNAs displayed strongly overlapping distribution patterns supporting the idea that these molecules interact to form a functional receptor (Peluso et al., 2004). Our data indicate that Paqr7 and Paqr8 may be important in sensory relay systems and other less well understood P₄ functions regulated by thalamic and cortical brain regions. Our findings suggest that P₄ may act through a number of signaling molecules to influence a much broader array of physiological effects than previously recognized.

The specific neural mechanisms underlying the role of Pgrmc1 have not yet been delineated, but the distinctive and overlapping patterns of Pgrmc1 and Serbp1 mRNAs suggest that these molecules may interact in neurons as they do in other cells. In granulosa cells, P₄ activation of the Pgrmc1/Serbp1 complex stimulates phosphorylation cascades through activation of protein kinase G (Peluso et al., 2007). Protein kinase G can activate additional kinases such as phosphoinositide 3-kinase, resulting in phosphorylation of Akt (Kandel and Hay, 1999, Brazil and Hemmings, 2001). The rapid phosphorylation of Akt in response to P₄ has been observed in cells from cortex and hippocampus (Singh, 2001, Hwang et al., 2009), and our study shows that both brain regions contain Pgrmc1 and Serbp1. Taken together, these observations suggest that phosphorylation events initiated by P₄ may involve the Pgrmc1/Serbp1 complex.
Brain regions rich in Pgrmc1 and Serbp1, as well as Pgrmc2 and Pgr, include the AVPV, medial preoptic (MPN), Arc and suprachiasmatic nuclei (SCN). These findings are in agreement with previous immunocytochemical and ISHH studies of Pgr and Pgrmc1 (Parsons et al., 1982, Krebs et al., 2000, Curran-Rauhut and Petersen, 2002, Meffre et al., 2005). Each of these nuclei contribute to the timing and magnitude of the preovulatory luteinizing hormone (LH) surge, a process in which P₄ exerts rapid and delayed effects. For example, estrogen induction of the LH surge requires Pgr expression in the AVPV/MPN (Chappell and Levine, 2000), and P₄ rapidly advances and augments the LH surge in estradiol-primed female rats (Krey et al., 1973, DePaolo and Barraclough, 1979, Levine and Ramirez, 1980). Our findings that the AVPV/MPN displayed high levels of Pgrmc1, Serbp1 and Pgrmc2 support the idea that these signaling molecules mediate rapid P₄ effects in the LH surge mechanism. P₄ also rapidly represses basal LH levels, and this action may involve the Arc (Banks and Freeman, 1978, Goodman et al., 1981, Richter et al., 2005), where Pgrmc1, Pgrmc2 and Serbp1 mRNA levels were also high. Finally, levels of these mRNAs were elevated in the SCN, and while not directly linked to reproduction, this nucleus sends projections to the AVPV and is critical for the temporal regulation of the LH surge (Paxinos and Watson, 1982, Watts and Swanson, 1987, de la Iglesia et al., 1995). Although the rapid effects of P₄ on LH surge release may be mediated by membrane-associated Pgr (Hammes and Levin, 2007), the current observations suggest that Pgrmc1, Pgrmc2 and Serbp1 may also play a role.

We found high levels of mRNAs encoding Pgrmc1, Pgrmc2, Serbp1 and Pgr in the VMH and in PAG, brain regions important for P₄-dependent female sex behaviors.
(Pfaff and Sakuma, 1979, Rubin and Barfield, 1983). This confirms a previous report of Pgrmc1 in the VMH (Krebs et al., 2000). Rat copulatory behavior requires activation of the Pgr within the VMH (Pollio et al., 1993, Ogawa et al., 1994), a nucleus that sends projections to many brain regions including the PAG (Saper et al., 1976). In the PAG, P4 rapidly elicits female sexual receptivity (Gorski, 1974, Wise et al., 1981), an effect originally attributed to Pgr expression in this region. However, work in Pgr-null mice shows that sexual receptivity can be rapidly induced by midbrain injection of P4, indicating a role for other signaling mechanisms (Beyer et al., 1988, Frye and Vongher, 1999). One such mechanism involves P4 metabolism to allopregnanolone, a molecule that induces feminine sex behaviors when injected into the midbrain (Beyer et al., 1988, Pfaff et al., 1994). The present work suggests another possibility, namely that Pgrmc1 contributes to elicitation of sexual receptivity by mediating rapid P4 signaling through phosphorylation events as described above. Alternatively, Pgrmc1 may influence sex behavior through actions on neurosteroid production in the midbrain, a process that may be required for feminine sex behavior (Akesson et al., 1988, Frye et al., 2007).

Consistent with this idea, Pgrmc1 interacts with key regulators of cholesterol homeostasis (Suchanek et al., 2005) and enhances steroidogenesis in diverse cell types (Laird et al., 1988, Min et al., 2004).

The present study identified high levels of Pgrmc1, Pgrmc2 and Serbp1 mRNAs in regions not directly linked to reproduction. For example, we confirmed previous findings that Pgrmc1 is expressed in the PVN and SON (Krebs et al., 2000, Meffre et al., 2005), and extend these findings to show that Pgrmc2 and Serbp1 mRNAs were also found in these nuclei. The physiological significance of these findings are unclear, but
Meffre *et al.* found that Pgrmc1 protein was expressed specifically within vasopressinergic neurons of the PVN and SON, suggesting that Pgrmc1 may play a role in regulation of water and ion homeostasis (Meffre *et al.*, 2005). We also found Pgrmc1, Pgrmc2 and Serbp1 mRNAs in CA1, CA2, CA3 and dentate gyrus. These findings may be relevant to the observation that P$_4$ enhances hippocampal-dependent cognitive performance (Hoshina *et al.*, 1994, Sandstrom and Williams, 2001). Our data also compliment previous findings of Pgrmc1 and Pgrmc2 mRNAs in neuroprogenitor cells derived from adult hippocampus (Liu *et al.*, 2009). In these cells, the authors showed that P$_4$ induces neurogenesis through Pgrmc1-dependent phosphorylation cascades that do not require Pgr. These findings suggest that non-genomic signaling events involving Pgrmc1, and possibly Pgrmc2 and Serbp1, underlie P$_4$ actions in diverse neural functions.

Neither *Paqr7* nor *Paqr8* genes were strongly expressed in neural regions that control gonadotropin release and contain abundant Pgrmc1 mRNA. This was somewhat surprising because these genes are colocalized in other parts of the reproductive system. For example, Paqr7, Paqr8 and Pgrmc1 have been detected in cells of the ovary, uterus, and placenta in rats, sheep, and humans (Zhu *et al.*, 2003a, Cai and Stocco, 2005, Peluso *et al.*, 2006, Zhang *et al.*, 2008, Ashley *et al.*, 2009). Moreover, in the rat corpus luteum, the expression of each of these genes changes during pregnancy, and the patterns of change for *Paqr7* and *Pgrmc1* expression are strikingly similar (Cai and Stocco, 2005). In contrast to Pgrmc1, neither Paqr7 nor Paqr8 mRNA levels are found at levels much above background in AVPV/MPN. However, the AVPV provides afferent projections to GnRH neurons and results of recent studies show that Paqr7 and
Paqr8 are functional in GT1-7 immortalized GnRH neurons (Sleiter et al., 2009). Thus, it is possible that Paqr7 and Paqr8 impact the neural control of reproduction, but through GnRH neurons scattered in the rostral preoptic area that also receive signals from Pgrmc1-containing neurons of the AVPV.

Though we did not find strong evidence for Paqr7 or Paqr8 gene expression in neuroendocrine nuclei, their expression was high in thalamic and cortical brain regions suggesting that these may participate in less well-studied actions of P4. The thalamus is known primarily as a sensory relay system but few studies have examined P4 actions within this brain region. However, P4 binding occurs in the lateral septal nucleus of the thalamus (Shughrue et al., 1992) where we found high levels of mRNA encoding Paqr8. This is important because present and previous studies have found that the Pgr is absent in this brain region (Parsons et al., 1982). P4 binding within the lateral septum decreases anxiety and depression-like behaviors (Pesold and Treit, 1992, Estrada-Camarena et al., 2002), and these effects have been presumed to be mediated by P4 metabolites acting on GABA_A receptors (Majewska et al., 1986). Similarly, GABA_A receptors are implicated in the neuroprotective effects of P4 in the cortex, a region in which we observed high levels of Paqr7, Paqr8 and Pgr mRNAs. In light of the present findings, it is also possible that Paqr8 mediates P4 actions in the lateral septum and cortex. This idea is supported by findings that in the cortex, maximal neuroprotective effects of P4 require activation of MAPK and phosphoinositide 3-kinase pathways (Kaur et al., 2007), suggesting a role for membrane-initiated P4 signaling, possibly involving Paqr7 and Paqr8.
In summary, we found that genes encoding Pgrmc1, Serbp1, Pgrmc2 and Pgr, but not Paqr7 and Paqr8, were highly expressed in many preoptic and hypothalamic nuclei and their projection sites. In contrast, Paqr7 and Paqr8 mRNA levels were most robust in thalamic nuclei and cortex. In view of these neuroanatomical findings, it is tempting to speculate that Pgrmc1 and Pgrmc2 control neuroendocrine functions, while Paqr7 and Paqr8 regulate affect and cognition. Additionally, the extensive overlap of Pgrmc1, Pgrmc2 and the classical Pgr mRNAs raise the intriguing possibility that these molecules interact within the same cells to regulate rapid and delayed P_4 effects in the brain. However, there were some regions in which Pgrmc1 and Pgrmc2 may exert effects independently of the Pgr. Together, these data provide anatomical information that will be important for determining the functional roles of these novel P_4 signaling molecules.
### 2.6.1: Sequences for Oligodeoxynucleotidyl Probes Used in ISHH Studies

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<tr>
<th>NCBI Gene and Accession #</th>
<th>Oligodeoxynucleotide Sequences 5’- 3’</th>
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2.6.2: Oligodeoxynucleotidyl Sequences for ISHH Validation Studies

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2.6.3: Localization and Abundance of mRNA encoding Pgr, Pgrmc1, Serbp1, Pgrmc2, Paqr7 and Paqr8 in Female Rat Forebrain

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<th>Pgrmc2</th>
<th>Paqr7</th>
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1 The ratings reflect the relative signal strength for each probe.
2.7.1 Photomicrographs of ISHH

Figure 2.7.1: Photomicrographs of film autoradiograms of hybridized rat brain sections to $^{33}$P-labeled oligodeoxynucleotidyl probes for (A) Pgrmc1, (B) Serbp1, (C) Pgrmc2, (D) Paqr7, (E) Paqr8 and (F) Pgr. For Pgrmc1, Serbp1, Pgrmc2, Paqr8 and Pgr, the sense (S) strand probe is shown in the third image of each series. For Paqr7, the section was hybridized to the antisense probe and then treated with RNAse A. Scale bar = 200 µm.
2.7.2 Photomicrographs of ISHH

Figure 2.7.2: Rostral to caudal arrangement of photomicrographs of film autoradiograms of hybridized rat forebrain sections to $^{33}$P-labeled oligodeoxynucleotidyl probes for (A) Pgrmc1, (B) Serbp1, (C) Pgrmc2, (D) Paqr7, (E) Paqr8 and (F) Pgr. Scale bar = 200 µm.
2.7.3 Photomicrographs of ISHH

Figure 2.7.3: Rostral to caudal arrangement of photomicrographs of film autoradiograms of hybridized rat forebrain sections to $^{33}$P-labeled oligodeoxynucleotidyl probes for (A) Pgrmc1, (B) Serbp1, (C) Pgrmc2, (D) Paqr7, (E) Paqr8 and (F) Pgr. Scale bar = 200 µm.
2.8 References


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Fernandes, M. S., Brosens, J. J. and Gellersen, B., 2008. Honey, we need to talk about the membrane progestin receptors. Steroids. 73, 942-952.


CHAPTER 3

17B-ESTRADIOL AND PROGESTERONE REGULATE PROGESTIN SIGNALING MOLECULES IN THE ANTEROVENTRAL PERIVENTRICULAR NUCLEUS, VENTROMEDIAL NUCLEUS AND SEXUALLY DIMORPHIC NUCLEUS OF THE PREOPTIC AREA IN RATS

3.1 Abstract

Recent work identified novel progestin signaling molecules, including progesterone receptor membrane component 1 (Pgrmc1), Pgrmc2, serpine mRNA binding protein 1 (Serbp1), progestin and adiponectin receptors 7 (Paqr7) and Paqr8. These molecules mediate rapid progesterone (P₄) effects in non-neural tissue and we recently mapped their expression in the brain. Many rapid effects of P₄ require 17β-estradiol (E₂) and P₄ priming; therefore, we examined the effects of ovarian hormones on the expression of these non-classical progestin signaling molecules. We focused specifically on the anteroventral periventricular nucleus (AVPV), the sexually dimorphic nucleus of the preoptic area (SDN-POA) and the ventrolateral portion of the ventromedial nucleus (VMNvl). These brain nuclei are important for female reproduction. Ovariectomized adult female rats were implanted with capsules containing sesame oil or E₂, and injected 48 hours later with sesame oil or P₄. Brains were collected eight hours later and RNA was isolated from the AVPV, SDN-POA and VMNvl. We assessed the effects of ovarian hormones on mRNA levels using quantitative polymerase chain reaction (QPCR). In the AVPV, Serbp1 mRNA levels were increased by P₄ in the presence of E₂, and Paqr8 was downregulated by P₄ alone. In the SDN-POA, combined E₂ and P₄ increased Pgrmc1 and Serbp1 mRNA levels, and
E₂ alone increased Paqr8 mRNA levels. Finally, in the VMNvl, P₄ increased mRNA levels encoding Pgrmc1, Pgrmc2 and Serbp1, and the combination of E₂ and P₄ increased Pgrmc1 and Serbp1 mRNA levels. Paqr7 was not regulated by E₂ or P₄ in any brain region examined. In summary, we showed that ovarian hormones regulate novel progestin signaling molecules in brain regions important for the neuroendocrine control of reproduction.

3.2 Introduction

P₄ signaling in the female brain regulates several facets of reproduction including the neural control of ovulation and the expression of feminine sex behaviors. The molecular mechanisms underlying these P₄ actions have been primarily attributed to activation of the progestin receptor (Pgr), a ligand-dependent transcription factor. This classical model of steroid hormone action has been revised to include rapid non-genomic effects of Pgr activation on diverse signaling systems, such as MAPK and c-Src pathways (Richer et al., 1998, Boonyaratanakornkit et al., 2001). However, this model may still be incomplete because many cells that lack Pgr retain rapid P₄-elicited responses (Ehring et al., 1998, Bar et al., 2000, Frye et al., 2006). These findings may be explained by recent discoveries of novel progestin signaling molecules that mediate diverse responses to P₄ in non-neural tissues (Falkenstein et al., 1999, Zhu et al., 2003b, Peluso et al., 2004).

We recently mapped the expression of several of these progestin signaling molecules in the rat forebrain (Intlekofer and Petersen, 2010). Genes encoding Pgrmc1, Pgrmc2 and Serbp1 were particularly abundant in neuroendocrine nuclei important for female reproduction. We confirmed and extended findings on the distribution pattern of
Pgrmc1 (Krebs et al., 2000, Sakamoto et al., 2004, Meffre et al., 2005), and showed that the pattern overlaps closely with that of its binding partner, Serbp1, and Pgrmc1 homologue, Pgrmc2. Few studies have examined the role of these molecules in neural function, but Pgrmc1 and Serbp1 have been implicated in the rapid effects of P₄ observed in ovarian cells and sperm (Correia et al., 2007, Peluso et al., 2009). While Pgrmc2 has not been studied in the context of rapid P₄ signaling, it may mediate P₄ actions in the ovary (Nilsson et al., 2006). Together these findings suggest that Pgrmc1, Pgrmc2 and Serbp1 may mediate non-classical P₄ signaling in the brain as in other tissues.

Other P₄ signaling molecules include Paqr7 and Paqr8, G-protein-like receptors that bind P₄ and regulate cAMP levels in several fish species (Zhu et al., 2003a, Zhu et al., 2003b, Hanna et al., 2006). Although controversy surrounds their role in mammalian cells (Fernandes et al., 2008), mRNAs encoding Paqr7 and Paqr8 have been detected in mammalian reproductive tissues (Zhu et al., 2003a). Our recent work showed that Paqr7 and Paqr8 gene expression is present in the hypothalamus (Intlekofer and Petersen, in press), though expression appears lower compared with that of Pgrmc1, Pgrmc2 and Serbp1. Other evidence suggests that Paqr7 and Paqr8 mediate P₄ signaling and couple to inhibitory G proteins in immortalized gonadotropin-releasing hormone (GnRH) neurons (Sleiter et al., 2009). Despite these significant advances, neither the regulation nor the functions of Paqr7 and Paqr8 in the brain are known.

In the female rodent, many of the rapid P₄ signaling events require 17β-estradiol (E₂) activation of estrogen receptor 1 (Esr1) (Edwards, 2005). This is partially due to E₂ induction of Pgr (Kastner et al., 1990), a nuclear transcription factor that also
activates rapid intracellular kinase cascades (Leonhardt et al., 2003). In regions of high Esr1 expression, such E2-induced effects result in greater P4-sensitivity. For example, in the preoptic area (POA) and ventromedial nucleus (VMN) of the hypothalamus, E2 exposure lowers cell signaling activation thresholds for P4, resulting in greater P4-sensitivity (Balasubramanian et al., 2008). In view of our recent findings that Pgrmc1, Pgrmc2, Serbp1, Paqr7 and Paqr8 are found in regions that contain Esr1 and Pgr, it is possible that E2 and/or P4 regulation of these molecules may be important in non-classical P4 signaling. This idea is supported by findings that Pgrmc1 expression is regulated by P4 in E2-primed rats (Krebs et al., 2000), uterine levels of Pgrmc2 mRNA vary across the estrus cycle (Zhang et al., 2008) and Paqr7 and Paqr8 ovarian expression is regulated by E2 (Karteris et al., 2006). It is unclear whether Pgrmc1 is regulated by steroids in brain regions other than the VMN, and no studies have tested the effects of ovarian steroids on Pgrmc2, Serbp1, Paqr7 and Paqr8 in the brain.

To address these issues, we examined the effects of E2, P4 and the combination of E2 and P4 (E2+P4) on levels of mRNA encoding Pgrmc1, Pgrmc2, Serbp1, Paqr7 and Paqr8. We focused specifically on the AVPV, the sexually dimorphic nucleus of the POA (SDN-POA) and the ventrolateral portion of the VMN (VMNvl). These nuclei have abundant expression of Esr1 and Pgr, are sexually dimorphic and are important for female reproduction (Dugger et al., 2007, Sakuma, 2009). In addition, we recently found expression of Pgrmc1, Pgrmc2, Serbp1, Paqr7 and Paqr8 in these nuclei (Intlekofer and Petersen, in press). We now report that ovarian steroid hormones regulate these putative progestin signaling molecules, and do so in a region-specific manner.
3.3 Materials and Methods

3.3.1 Animals

All protocols and post-operative care were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts, and animals were housed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Twenty-eight adult female Sprague-Dawley rats (200-250 g, Harlan, Madison, WI) were housed individually on a 14:10 light:dark cycle with food and water provided ad libitum. Animals were anesthetized with isofluorane and bilateral ovariectomies were performed through very small (5-mm) flank incisions that minimized tissue trauma. I observed animals for respiratory distress and bleeding for 6 h postoperatively and examined again at 12, 24 and 48 h to ensure that they were freely moving and had no significant weight loss. One week later (Day 0), animals were implanted s.c. with Silastic capsules (Dow Corning, Midland, MI; 1.57 mm, o.d., 3.8 mm; 30 mm length) containing either sesame oil vehicle or E2 (150 µg/ml 17β-estradiol in sesame oil) as described previously (Petersen and LaFlamme, 1997). At 0900 H on Day 2, animals were injected s.c. with either sesame oil vehicle or 50 mg P4. Eight hours later, animals were anesthetized with CO₂ and brains were rapidly frozen on powdered dry ice, wrapped in Parafilm (American Can Co., Greenwich, CT) and stored at -80 C.

3.3.2 Tissue preparation

Coronal cryosections that contained the AVPV, SDN-POA and VMNvl were acquired using a Leica CM3000 cryostat (Nussloch, Germany). These sections were taken from the rostral AVPV (bregma -0.02 mm), SDN-POA (bregma -0.4 mm) and
VMNvl (bregma -0.20 mm) (Swanson, 1998). I obtained tissue punches from these sections using a 1.0-mm diameter Harris Uni-Core™ tissue needle (Ted Pella Inc., Redding, CA) from a single 300-µm section as illustrated in Fig. 3.7.1, and used this tissue for quantitative polymerase chain reaction (QPCR).

### 3.3.3 RNA Isolation and QPCR

RNA was isolated from tissue punches using the RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed with the QuantiTect Reverse Transcription Kit (Qiagen), using manufacturer’s protocol. QPCR was performed in a Stratagene Mx3000P thermocycler (Agilent Technologies, Wilmington, DE) programmed as follows: 95 C, 10 min; 40 cycles of 95 C for 15 sec; and 60 C for 60 sec. Reactions contained reagents from QuantiTect SYBR Green Kit, following manufacturer’s protocol (Roche Diagnostics, Indianapolis, IN). Specific primer sets were obtained from Integrated DNA Technologies (Coralville, Iowa), and sequences are listed in Table 3.6. The efficiency of each primer set was validated over a range of cDNA concentrations and samples with no cDNA were included as negative controls. Primer specificity was verified using melting curve analyses and confirmation of a single fluorescence peak in each QPCR reaction. Melting curve analyses were performed by heating samples to 95 C for two min, 55 C for 15 sec, and recording fluorescence measurements during incremental increases of 0.5 C for 80 cycles. Primer specificity was also validated using 2% agarose gel electrophoresis to verify single products following the QPCR reaction. Fluorescence measurements were detected using MxPro™ QPCR analysis software (Agilent Technologies). I verified that levels of mRNA encoding β-actin did not differ among treatments; therefore, I used it as an internal control (primers as
indicated in Table 3.6.1). The ΔΔCt method was used to analyze the data (Livak and Schmittgen, 2001).

3.3.4 Statistics

All data are expressed as mean ± SEM. Effects of E₂, P₄ and E₂+P₄ on mRNA levels were detected using a one-way ANOVA, followed by pair-wise comparisons using t-tests with Bonferroni correction.

3.4 Results

3.4.1 QPCR Reaction Specificity

First, melting curve analyses verified a single peak of fluorescence, and the size of each product was confirmed by gel electrophoresis. In addition, the PCR amplification efficiency calculated from the standard curve was between 96-100% for all primer sets used. Consistent with our previous in situ hybridization findings (Intlekofer and Petersen, in press), QPCR verified that the genes of interest were expressed in the AVPV, SDN-POA and VMNvl.

3.4.2 Esr1 and Pgr mRNA Levels

In ovariectomized adult rats, exposure to E₂ reduced Esr1 mRNA levels in the AVPV, SDN-POA and VMNvl (Fig. 3.7.2 a, b and c, respectively). P₄ administration significantly decreased Esr1 mRNA levels in the AVPV (Fig. 3.7.2a), but not in the SDN-POA (Fig. 3.7.2b) or VMNvl (Fig. 3.7.2c). In all three areas examined, E₂+P₄ reduced levels of mRNAs encoding Esr1 (Fig. 3.7.2a, b and c). E₂ markedly increased levels of mRNA encoding Pgr in all brain regions examined (Fig. 3.7. 2d, e and f), and in the AVPV and SDN-POA, E₂+P₄ increased Pgr mRNA levels.
3.4.3 Pgrmc1, Serbp1 and Pgrmc2 mRNA Levels

In the AVPV, mRNA levels of the homologues Pgrmc1 and Pgrmc2 were unaltered by treatment with E$_2$, P$_4$ or E$_2$+P$_4$ (Fig. 3.7.3a and d). Within the SDN-POA, Pgrmc1 and Pgrmc2 levels were not significantly affected by E$_2$ or P$_4$ alone, but E$_2$+P$_4$ increased Pgrmc1 (Fig. 3.7.3b) and decreased Pgrmc2 mRNA levels (Fig. 3.7.3e). In the VMNvl, P$_4$ increased Pgrmc2 mRNA levels (Fig. 3.7.3f).

Levels of mRNA encoding Pgrmc1 and its binding partner, Serbp1, were increased by P$_4$ or E$_2$+P$_4$ in the VMNvl (Fig. 3.7.3c and i). In the SDN-POA, only E$_2$+P$_4$ increased Pgrmc1 and Serbp1 mRNA levels (Fig. 3.7.3b and h). In the AVPV, E$_2$+P$_4$ increased Serbp1 (Fig. 3.7.3g), but not Pgrmc1 mRNA levels (Fig. 3.7.3a).

3.4.4 Paqr7 and Paqr8 mRNA levels

Ovarian steroids did not regulate Paqr7 mRNA levels in any brain region examined (Fig. 3.7.4a, b and c). In contrast, Paqr8 mRNA levels were repressed by treatment with P$_4$ in the AVPV (Fig. 3.7.4d), increased by E$_2$ in the SDN-POA (Fig. 3.7.4e), but not altered in the VMNvl (Fig. 3.7.4f).

3.5 Discussion

These results are the first to show that E$_2$ and P$_4$ regulate non-classical progestin signaling molecules in the AVPV, SDN-POA and VMNvl. Significantly, E$_2$+P$_4$ increased Serbp1 mRNA levels in all brain regions examined, but increased expression of its putative binding partners, Pgrmc1 and Pgrmc2, in a region-specific manner. These findings are consistent with the idea that Serbp1 availability is the key factor determining P$_4$ responsiveness of Pgrmc1 complexes (Peluso et al., 2004). Ovarian steroid regulation of Paqr8 also varied by brain region; however, the closely-related
Paqr7 was not regulated in any region examined. Together these findings support the hypothesis that Pgrmc1, Pgrmc2, Serbp1 and Paqr8 mediate rapid P₄ signaling within neuroendocrine nuclei important for female reproduction.

Our findings that E₂ decreased Esr1 and increased Pgr in the AVPV and VMNvl are similar to results of previous studies (Lauber et al., 1990, Simerly and Young, 1991). We now report that the same pattern exists in the SDN-POA. Interestingly, in the VMNvl, P₄ abrogated the effects of E₂ on Pgr mRNA levels. Although this was not seen in other brain regions examined herein, P₄ blocks E₂ induction of Pgr in non-neural cells (Kraus and Katzenellenbogen, 1993).

We found that E₂+P₄ increased Pgrmc1 mRNA levels in the SDN-POA but not the AVPV; however, in both regions Serbp1 expression was increased, and this may be sufficient for rapid P₄ effects. This idea is supported by findings that P₄ responses mediated by the Pgrmc1/Serbp1 complex depend upon Serbp1 levels in non-neural cells (Peluso et al., 2005). Interestingly, E₂+P₄ increased Pgrmc2 mRNA levels in the AVPV, though no studies have tested whether Serbp1 binds Pgrmc2 to form a functional complex. These mechanisms are of particular interest as the AVPV is required for induction of the preovulatory luteinizing hormone surge (Wiegand et al., 1980, Ronneklev and Kelly, 1986, Petersen et al., 1995, Chappell and Levine, 2000). The AVPV and SDN-POA have dense projections to GnRH neurons (Simonian et al., 1999), and show abundant Pgrmc1, Pgrmc2 and Serbp1 mRNA levels (Intlekofer and Petersen, 2011). Thus, further studies are warranted to determine whether these signaling molecules mediate rapid P₄ effects in the AVPV and SDN-POA.
P₄ and E₂+P₄ increased Pgrmc1 and Serbp1 mRNA levels in the VMNvl, a region in which P₄ facilitates lordosis (Pfaff and Sakuma, 1979, Pfaff et al., 1994, Frye and Vongher, 1999, Frye, 2001). These rapid P₄ effects are partially due to activation of cGMP-dependent protein kinase (DeBold and Frye, 1994, Lydon et al., 1995). This is especially interesting because Pgrmc1 is involved in P₄ induction of cGMP-dependent protein kinase (Peluso and Pappalardo, 2004), and the C-terminus of Pgrmc1 contains several putative kinase binding sites (Cahill, 2007). Other researchers examining the entire VMN also found that Pgrmc1 was regulated by ovarian steroids; however, in that study E₂ alone increased Pgrmc1 mRNA levels (Krebs et al., 2000). Factors that may explain these differences include dosage, duration of treatment, and region examined. Overall, these findings suggest a link between Pgrmc1/Serbp1 and the rapid facilitation of feminine sex behavior.

Our findings are the first to show that ovarian steroids regulate Paqr8 mRNA levels in the brain. Similar to studies in myometrial cells, we found that E₂ increased both Paqr8 and Pgr mRNA levels in the SDN-POA. These results are interesting in light of evidence that Paqr8 cross-talks with Pgr through coupling to inhibitory G-proteins and decreasing Pgr transactivation (Karteris et al., 2006). In contrast to Paqr8, ovarian steroid exposure did not alter Paqr7 mRNA levels in any brain region examined. In other reproductive tissues, E₂ and P₄ also have variable effects on these signaling molecules (Cai and Stocco, 2005, Fernandes et al., 2005). Thus, despite their structural similarities, Paqr7 and Paqr8 may be regulated differently by ovarian steroids and may have functionally distinct roles.
Though reproductive functions coordinated by ovarian hormones have been studied extensively, the underlying molecular events are unclear. The present studies identified several steroid-inducible progestin signaling molecules that may mediate rapid P₄ actions in the neuroendocrine control of reproduction. The functional relevance and specific role(s) of these novel signaling molecules will be the topic of future research.
### 3.6.1: Primers Used in QPCR Studies

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<td>76</td>
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<td></td>
<td>GCGGCAGTGGCCATCTC</td>
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<td></td>
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<tr>
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<td>214</td>
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<tr>
<td></td>
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3.7.1 Diagram of Brain Sections

Figure 3.7.1: Diagrams of brain sections containing the a) AVPV, b) SDN-POA and c) VMNvl modified from the atlas of Swanson (1998). Circles indicate regions from which tissue was excised for analysis. OC, optic chiasm; 3V, third ventricle; AC, anterior commissure.
3.7.2 Esr1 and Pgr mRNA Levels

Figure 3.7.2: Levels of mRNAs encoding Esr1 and Pgr in ovariectomized rats treated with oil, E₂, P₄ or E₂+P₄. Esr1 mRNA levels in the AVPV (a), SDN-POA (b) and VMNvl (c), and Pgr mRNA levels in the AVPV (d), SDN-POA (e) and VMNvl (f) were determined by QPCR. Bars = means ± SEM. a Significantly different from oil-treated controls; b significantly different from E₂-treated animals; c significantly different from P₄-treated animals; values considered significantly different if p < 0.05 in post-hoc analyses. One-way ANOVA results: a) F(3,22) = 4.72, p < 0.010; b) F(3,20) = 11.81, p < 0.0002; c) F(3,20) = 8.14, p < 0.001; d) F(3,20) = 20.83, p < 0.0001; e) F(3,20) = 17.14, p < 0.0001; f) F(3,20) = 41.49, p < 0.0001.
3.7.3 Pgrmc1, Serbp1 and Pgrmc2 mRNA Levels

Figure 3.7.3: Levels of mRNAs encoding Pgrmc1, Pgrmc2 and Serbp1 in ovariectomized rats treated with oil, E\textsubscript{2}, P\textsubscript{4} or E\textsubscript{2}+P\textsubscript{4}. Pgrmc1 mRNA levels in the AVPV (a), SDN-POA (b) and VMNvl (c), Pgrmc2 mRNA levels in the AVPV (d), SDN-POA (e) and VMNvl (f), and Serbp1 mRNA levels in the AVPV (g), SDN-POA (h) and VMNvl (i) were determined by QPCR. Bars = means ± SEM. a significantly different from oil-treated controls; b significantly different from E\textsubscript{2}-treated animals; c significantly different from P\textsubscript{4}-treated animals; values considered significantly different if \( p < 0.05 \) in post-hoc analyses. One-way ANOVA results: a) F(3,22) = 2.32, \( p < 0.103 \); b) F(3,20) = 8.56, \( p < 0.001 \); c) F(3,22) = 28.59, \( p < 0.0001 \); d) F(3,22) = 21.79, \( p < 0.0001 \); e) F(3,22) = 6.56, \( p < 0.002 \); f) F(3,22) = 21.13, \( p < 0.0001 \); g) F(3,22) = 4.65, \( p < 0.01 \); h) F(3,22) = 4.55, \( p < 0.01 \); i) F(3,22) = 6.16, \( p < 0.003 \).
3.7.4 Paqr7 and Paqr8 mRNA Levels

Figure 3.7.4: Levels of mRNAs encoding Paqr7 and Paqr8 in ovariectomized rats treated with oil, E2, P4 or E2+P4. Paqr7 mRNA levels in the AVPV (a), SDN-POA (b) and VMNvl (c), and Paqr8 mRNA levels in the AVPV (d), SDN-POA (e) and VMNvl (f) were determined by QPCR. Bars = means ± SEM. a Significantly different from oil-treated controls; b significantly different from E2-treated animals; c significantly different from P4-treated animals; values considered significantly different if \( p < 0.05 \) in post-hoc analyses. One-way ANOVA results: a) \( F(3,22) = 0.65, p < 0.59 \); b) \( F(3,22) = 0.037, p < 0.99 \); c) \( F(3,20) = 3.67, p < 0.03 \); d) \( F(3,22) = 10.39, p < 0.0002 \); e) \( F(3,22) = 33.87, p < 0.0001 \); f) \( F(3,20) = 1.35, p < 0.29 \).
3.8 References


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CHAPTER 4

DUAL-LABEL IN SITU HYBRIDIZATION REVEAL THAT PGRMC1 AND SERBP1 ARE COLOCALIZED WITHIN GABAERGIC NEURONS OF THE ANTEROVENTRAL PERIVENTRICULAR NUCLEUS OF THE FEMALE RAT

4.1 Abstract

The neural control of ovulation requires generation of the gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) surges by 17β-estradiol (E$_2$) and progesterone (P$_4$). While the majority of research in this field concerns the essential role of E$_2$, the role of P$_4$ in the preovulatory surge is less understood. Numerous studies in rats have shown that P$_4$ controls the timing and amplification of the LH surge. While the exact neurobiological mechanisms that underlie these actions remain unclear, they are likely to occur through P$_4$ action in the preoptic area and hypothalamus, brain regions with abundant classical progesterone receptors (Pgr). We recently found that these regions also express high levels of progesterone signaling molecules, including Progesterone Receptor Membrane Component 1 (Pgrmc1) and Serpine mRNA Binding Protein 1 (Serbp1), indicating that these additional molecules may subserve progesterone actions in the preovulatory surge. To begin to test this idea, we used dual-label in situ hybridization to determine whether these progesterone signaling molecules are colocalized in neurons critical for the LH surge mechanism. We now report that the majority of neurons important for the neural control of ovulation contain Pgrmc1 and Serbp1 mRNA, and both signaling molecules are colocalized in the same neurons. Together, these findings indicate that progesterone actions in regulation of the LH surge may involve progesterone signaling molecules, Pgrmc1 and Serbp1.
In line with this idea, Pgr is abundantly expressed in the hypothalamus, but we and others have recently found progesterone signaling molecules in hypothalamic regions known to be important for generation of the LH surge.

4.2 Introduction

Although the regulation of the luteinizing hormone (LH) surge by 17β-estradiol (E$_2$) and progesterone (P$_4$) has been studied extensively, the molecular actions of these steroid hormones continue to emerge. In the neural control of the preovulatory surge, hormonal status is conveyed to gonadotropin releasing hormone (GnRH) neurons by neural projections that originate in the anteroventral periventricular nucleus (AVPV) (Simonian et al., 1999, Ottem et al., 2004). Previous findings show that these important AVPV neurons are dual-phenotype GABA/glutamate neurons (Ottem et al., 2004), and contain abundant E$_2$ and P$_4$ receptors (Simerly et al., 1990{Shughrue, 1997 #1045). While E$_2$ is a requisite hormone for the onset of the GnRH and LH surges, P$_4$ regulates more subtle aspects of the surge including the timing and amplification (Everett, 1948, Rothchild, 1965, DePaolo and Barraclough, 1979), though P$_4$ actions in this capacity are not well understood. New insights into P$_4$ mechanism of action have identified novel signaling molecules that may be involved in modulating the LH surge. In support of this idea, we recently reported that the AVPV contains progesterone receptor membrane component-1 (Pgrmc1) and binding partner Serpine mRNA binding protein 1 (Serbp1) (Intlekofer and Petersen, 2010). Thus, P$_4$ signaling molecules in the AVPV may mediate important P$_4$ effects in the neural control of the preovulatory LH surge.

Pgrmc1 and Serbp1 are binding partners that mediate P$_4$ actions in other tissues (Peluso et al., 2004, Peluso et al., 2008), but it is unclear whether they could play a
similar role in dual-phenotype neurons. While this possibility has not yet been explored, several lines of evidence support the idea that P₄ signaling molecules such as Pgrmc1 and Serbp1 could mediate P₄ actions in regulation of the surge. For example, membrane bindings sites for P₄ have been identified in the hypothalamus (Ke and Ramirez, 1990), and P₄ exerts rapid Pgr-independent effects (Frye and Vongher, 2001, Sleiter et al., 2009). Given the important role of GABA/glutamate neurons of the AVPV, and high expression of hormone receptors in the AVPV region, it is possible that additional P₄ signaling molecules account for the effects of P₄ on LH surge release. To address this issue, we used dual-label in situ hybridization histochemistry (ISHH) to determine whether AVPV GABA/glutamate neurons contain Pgrmc1 and Serbp1 and whether these signaling molecules are coexpressed by AVPV neurons.

4.3. Materials and Methods

4.3.1 Animals and Tissues

All animals were housed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and all protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts. We used brains from six adult female Sprague-Dawley rats (200-225 g, Harlan, Madison, WI). Animals were housed on a 14:10 light:dark cycle with free access to food and water, and ovariectomized under isofluorane anesthesia. A week later, the rats received s.c. implants of Silastic capsules (Dow Corning, Midland, MI; 1.57 mm, o.d., 3.8 mm; 30 mm length) containing E₂ (150 µg/ml in sesame oil) as previously described (Petersen et al., 1995). After two days, rat brains were collected at 1500 h and rapidly frozen on powdered dry ice. Brains were cryosectioned (Leica
CM3000, Nussloch, Germany) at 14 µm through the rostral preoptic area, a brain region that contains the AVPV. Sections were thaw-mounted onto gelatin-coated slides, dried briefly at 42°C on a slide warmer, and stored at -80°C until prehybridization.

4.3.2 Transcription Template Preparation

PCR-generated transcription templates for rat Pgrmc1 and Serbp1 were produced using rat cDNA and GoTaq® Green Master Mix and manufacturer’s protocol (Promega, Madison, WI). Specific primer sets were obtained from Integrated DNA Technologies (Coralville, IA) and sequences are listed in Table 4.6.1. Reactions were performed in an Eppendorf Thermocycler (Hauppauge, NY) programmed as follows: 95°C, 10 min, 40 cycles of 95°C for 15 sec and 60°C for 60 sec. PCR products were purified by gel electrophoresis and extracted using GenElute™ Agarose Spin-Columns and manufacturer’s protocol (Sigma-Aldrich, St. Louis, MO). The resulting cDNA fragments were inserted into vectors using the TOPO-TA® cloning kit (Invitrogen, Carlsbad, CA). One Shot Mach1 T1-phage resistant E.Coli (Invitrogen) were transformed with plasmids containing the cDNA fragments and plasmids were isolated with the QIAfilter Plasmid Mini kit (Qiagen). The identity and direction of each insert was confirmed by sequencing provided by Genewiz (South Plainfield, NJ.)

For detection of GABAergic cells, cDNA templates for Gad1 and 2 were produced as described previously (Hays et al., 2002). Primers used to generate templates for Pgr, Gad1 and Gad2 are specified in Table 4.6. To produce template for rat GnRH cRNA probes, I linearized a modified pSP65 plasmid containing a 330-bp cDNA fragment (provided by Dr. John Adelman, Vollum Institute, Portland, OR). This fragment corresponds to exons I-IV of mRNA encoding GnRH (Adelman et al., 1986).
Plasmids were linearized using restriction sites present within the vector and restriction enzymes (Promega). Linearized templates were extracted with phenol/chloroform, precipitated with 0.4 M NaCl₂ and ethanol and stored in 10 mM Tris and 1 mM EDTA (TE; pH 8.0) at -20°C until they could be used for transcription of cRNA probes.

4.3.3 Probe preparation

The cDNA templates were transcribed as described previously (Petersen et al., 1993) and all enzymes were provided by Promega. Briefly, I used a DNA SpeedVac (Savant, Farmingdale, NY) to dry 120 pmol (12 µM final concentration) of ³⁵S-UTP (PerkinElmer, Boston, MA). To generate radiolabeled probes for Pgrmc1 and Serbp1, I added the following in a total volume of 10 µl: 0.5 mM linearized template, 1X transcription buffer, 10 mM dithiothreitol (DTT), 20 U RNAsin, 3 µM UTP, 0.5 mM each ATP, CTP, and GTP and 10 U RNA polymerase. Each reaction was incubated for 30 min at 37°C, then a second 10 U RNA polymerase aliquot was added, mixed and incubated for 30 min at 37°C. Deoxyribonuclease I (20 U) was added to each reaction to degrade the DNA template in the presence of 20 U RNAsin. The transcription reaction volume was adjusted to 100 µl by addition of TE, 0.4 M NaCl₂ and 0.5 µl tRNA. I extracted cRNA probes with phenol-chloroform, removed unincorporated nucleotides by ethanol precipitation and resuspended them in 100 µl TE.

GABAergic cells were detected using a mixture of digoxigenin-labeled cRNA probes encoding Gad1 and 2 and prepared as previously described (Hays et al., 2002). I also generated digoxigenin-labeled probes to detect GnRH neurons as described previously (Sannella and Petersen, 1997). For both Gad and Serbp1 detection, I
produced digoxigenin-labeled probes using 1 µg linearized cDNA template, 1X transcription buffer, 500 µM each ATP, CTP and GTP, 50µM UTP, 10 mM DTT and 20 U RNA polymerase in the presence of 250 µM digoxigenin-11-UTP (Roche, Molecular Biochemicals, Indianapolis, IN). The mixture was incubated at 37°C for 1 h and then an additional 20 U RNA polymerase aliquot was added and incubation continued for another hour. I eliminated the DNA template using 20 U deoxyribonuclease I in the presence of 20 U RNasin. The reaction volume was adjusted to 100 µl by the addition of nuclease-free water, precipitated twice with 0.4 M NaCl₂ and ethanol, and then resuspended in 100 µl TE.

4.3.4 In situ hybridization

Tissue sections were prehybridized as previously described (Ottem et al., 2004). Nonisotopic and radiolabeled cRNA probes were added to hybridization buffer consisting of 50 % formamide, 1XSSC, 20 % dextran sulfate, 1X Denhardt's solution (0.02 % Ficoll-400, 0.02 % bovine serum albumin, 0.02 % polyvinylpyrrolidone-40), 500 µg/ml yeast tRNA, 500 µg/ml heparin sodium salt, 0.1 % sodium pyrophosphate and freshly prepared 400 mM DTT was added before use. Twenty µl of hybridization buffer containing cRNA probes (1.0 x 10⁶ cpm radioisotopic probe and 1 µl digoxigenin-labeled probe) was applied to each tissue section and covered with glass coverslips, and then sections were incubated for 16 h at 52°C. Additionally, representative sections were hybridized to radiolabeled sense probes for Pgrmc1 and Serbp1 in order to verify specificity. Specificity of probes for Gad 1 and 2 were determined previously (Ottem et al., 2004).
4.3.5 Signal detection

Posthybridization was performed on an orbital or lateral shaker at room temperature unless otherwise specified. Coverslips were removed and the slides were washed as follows: 50 % (v/v) formamide/2XSSC for 40 min at 52 °C; 2XSSC for 20 min; RNase buffer (0.5 M NaCl, 10 mM Tris; and 1 mM EDTA; pH 8.0) containing 25 µg/ml RNAse A (Roche) for 30 min at 37 °C; 2XSSC for 10 min; 50% (v/v) formamide/2XSSC for 20 min; and 2XSSC for 10 min. Next, I processed slides for immunocytochemical detection of digoxigenin.

Nonspecific binding was blocked by placing tissue sections in a solution containing 0.5% w/v blocking reagent (Roche) in maleate buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5) and 0.3% Triton X-100 for 1 h. Slides were washed twice for 5 min in 0.1 M Tris-HCl and 0.15 M NaCl (TN) and then incubated at 4 °C in 0.2 % w/v blocking reagent (Roche) and anti-digoxigenin antisera conjugated to horseradish peroxidase (Roche; 1:150) using probe clips (Midwest Scientific, PC200) without shaking. After 48 h, probe clips were removed and slides were washed in maleate buffer, TN, and TN with 0.05% TritonX; Sigma-Aldrich (TNT) for 5 min each. Slides were transferred to plastic mailers containing biotinylated tyramide from a Renaissance TSA-indirect ISH Kit (Perkin-Elmer; 1:250) in TNT for 10 min. Sections were washed in TNT three times for 3 min each and then incubated in ABC Elite (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) reagent for 30 min at 37 °C using probe clips and manufacturer’s protocol. Probe clips were removed and slides washed 3 times, 3 min each in TNT. Digoxigenin signal was visualized using TNT containing 1 mM diaminobenzidine (Sigma D5905) and 0.1 mM hydrogen peroxide. After specific signal
appeared, this reaction was halted by rinsing slides in 0.1 M Tris (pH 7.6) for 3 min followed by a brief rinse in water. Finally slides were dehydrated for 3 min in 70% ethanol and air-dried.

I detected radioisotopic probe signal autoradiographically using NTB emulsion (Kodak, Rochester, NY) and manufacturer’s protocol. Slides were later developed at 14° C using Kodak Dektol developer and Kodak fixer. After a rinse in water, I air-dried the slides and dipped them briefly in xylenes prior to coverslipping with Cytoseal 60™ mounting medium (Richard-Allan Scientific, Kalamzoo, MI). Images were acquired using a CCD videocamera (QImaging QICAM FAST color) and BioQuant Imaging Software (Nashville, TN).

4.3.6 Statistics

In order to analyze dual-label ISHH studies, I used four to six AVPV sections from each animal and determined the mean number of GAD-positive neurons containing Pgrmc1 and the mean number containing Serbp1 mRNA. This value was used to determine the percentage of GAD-positive neurons that contained specific signal for Pgrmc1 or Serbp1. Similarly, percentages of Serbp1-positive neurons that contained specific Pgrmc1 signal were also determined. Signal was considered specific if the digoxigenin-labeled neurons contained four-times more 35S-labeled probe signal than background. The grand means of each group were acquired from the means of individual animals.
4.4 Results

4.4.1 AVPV GABA Neurons Contain Pgrmc1 mRNA

Results of the dual-label ISHH studies showed that in E$_2$-primed ovariectomized female rats, AVPV neurons that were GAD-positive (as determined using digoxigenin-labeled cRNA probes for GAD1 and 2) contain Pgrmc1 mRNA (Figure 4.6.1a). Quantification studies determined that virtually all GABAergic neurons contain Pgrmc1 mRNA (Figure 4.6.1d).

4.4.2 AVPV GABA Neurons Contain Serbp1 mRNA

In the AVPV, neurons that contained GAD mRNA also contained Serbp1 mRNA (Figure 4.6.1b). Serbp1 mRNA was specifically detected in approximately 86% of GAD-positive neurons in ovariectomized E$_2$-primed rats (Figure 4.6.1d).

4.4.3 Pgrmc1 and Serbp1 mRNA are Coexpressed by AVPV Neurons

Dual-labeling experiments revealed that Pgrmc1 and Serbp1 mRNA are present within the same neurons in the AVPV of ovariectomized E$_2$-primed female rats (Figure 4.6.1c). Pgrmc1 (detected using a radioactively-labeled probe) was specifically expressed by approximately 94% of Serbp1+ neurons in ovariectomized E$_2$-primed rats (Figure 4.6.1d). A small percentage of AVPV neurons that were Serbp1+ did not contain detectable levels of Pgrmc1 signal.

4.5 Discussion

The present findings are the first to show that AVPV cells contain mRNA encoding both Pgrmc1 and binding partner Serbp1. Moreover, nearly all expression of these two genes was within GABAergic neurons (as determined by using GAD as a marker of GABAergic neurons). Given the importance of these GABAergic neurons in
relaying hormonal cues to GnRH neurons, our findings indicate that P₄ may exert
diverse actions through Pgrmc1 and Serbp1 in timing and amplifying the LH surge.
The finding that Pgrmc1 and Serbp1 mRNAs are contained within the same AVPV
neurons lends support to the notion that these binding partners form a functional
receptor complex to mediate P₄ actions in AVPV neurons as they do in granulosa cells
(Peluso et al., 2004, Peluso et al., 2006). Taken together, these data indicate that
Pgrmc1 and Serbp1 actions as P₄ signaling molecules may be important in the neural
control of ovulation.

The neural expression of Pgrmc1 and Serbp1 within GABAergic AVPV neurons
is intriguing as these neurons are critical for regulation of LH surge release. Ample
evidence supports the idea that GABA release from AVPV efferents restrains GnRH
firing and the onset of the surge. For example, GABA release within the vicinity of
GnRH cell bodies increases in the morning but falls before the afternoon onset of the
LH surge (Robinson et al., 1991, Jarry et al., 1995). In the AVPV, levels of Gad1
mRNA (marker of GABAergic neuronal activation), as well as the amount of vesicular
GABA transporter in synapses on GnRH neurons (Ottem et al., 2004) rise, then fall just
before LH surge release. These changes parallel those of GABA release into the
preoptic area on the day of the surge (Jarry et al., 1995) and a decline in GABA is
GnRH neurons express GABAₐ receptors (Petersen et al., 2003), and GABAₐ activation
hyperpolarizes GnRH neurons (Herbison, 1998, Han et al., 2002). Together, these
findings highlight the importance of AVPV GABergic neurons and underscore the
importance of this subset of neurons as a nodal point conveying hormonal status to
GnRH neurons. Thus, the present finding that P$_4$ signaling molecules are present on the vast majority of GABAergic neurons suggests that they may influence the regulatory input of AVPV dual-phenotype neurons to GnRH cell bodies.

The exact role of P$_4$ in the LH surge release is an issue that has been difficult to resolve as E$_2$ alone is sufficient to increase GnRH levels in the neural control of the LH surge (Rothfeld et al., 1989, Rosie et al., 1990). Therefore, our understanding on this topic is centered on the observation that P$_4$ coordinates the timing of the onset as well as increasing the magnitude of the LH surge (Everett, 1948, Rothchild, 1965, DePaolo and Barraclough, 1979). This positive-feedback action is largely attributed to E$_2$-induced Pgr activation (Chappell and Levine, 2000), a highly induced gene in the estrogen receptor-rich AVPV (Simerly et al., 1990, Shughrue et al., 1997). Our current findings suggest another alternative, namely that P$_4$ activation of Pgrmc1 and Serbp1 may contribute to the regulatory functions of P$_4$ on LH surge magnitude and timing. While further research concerning this possibility is warranted, evidence from other cells support the concept that Pgrmc1 and Serbp1 may mediate P$_4$ signaling in AVPV neurons.

Our finding that Pgrmc1 and Serbp1 are coexpressed within the same cells is in line with the idea that both are required for P$_4$ actions. Current research on these binding partners shows that depletion of Pgrmc1 or Serbp1 limits the ability of P$_4$ to maintain cell viability in granulosa cells (Peluso et al., 2004, Peluso et al., 2008). Additional studies have shown that Pgrmc1 overexpression increases [$^3$H]-P$_4$ binding and P$_4$-responsiveness (Peluso et al., 2006), indicating that Pgrmc1 mediates P$_4$ actions. This idea appears to hold for some neural cells, as a recent study reports that Pgrmc1 is
required for the proliferative effect of P₄ on hippocampal neuroprogenitor cells (Liu et al., 2009). Although the roles of Pgrmc1 and Serbp1 in mediating neural responses to P₄ remains unknown, our present findings raise the possibility that may be important in the AVPV for P₄-dependent amplification of LH surge release.
### 4.6.1: Primers Used in Template Production

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<th>Amplicon (bp)</th>
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<td>825</td>
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</tr>
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4.7.1 Pgrmc1 and Serbp1 are Coexpressed by GABAergic AVPV Neurons

Figure 4.7.1: Most GABAergic neurons (Gad-positive) of the adult female rat contain Pgrmc1 (A) and Serbp1 (B), and Pgrmc1 and Serbp1 are coexpressed within many of the same neurons (C). Dual-label ISHH used $^{35}$S-labeled cRNA probes (black grain) and digoxigenin-labeled cRNA probes (brown stain) and adult female rat brain sections containing the AVPV. Photomicrographs were produced using probes $^{35}$S-labeled for Pgrmc1 (A, C) and Serbp1 (B), in combination with digoxigenin-labeled GAD (marker of GABAergic neurons) (A, B), and Serbp1 (C). Quantification of colocalization experiments is shown in D. Scale bar = 50 µm.
4.8 References


CHAPTER 5
PROGESTERONE RECEPTOR MEMBRANE COMPONENT 1
COREGULATES NOVEL GENE TARGETS NR4A1, SPNA1, TGM2 AND IFIT3
AND REGULATES BASAL STAT3 ACTIVATION IN NEURAL CELLS

5.1 Abstract

Progesterone receptor membrane component 1 (Pgrmc1) is a signaling molecule important for progesterone actions in reproductive tissues. While much of the research regarding Pgrmc1 function has been conducted in ovarian cells, we and others have recently found high levels of Pgrmc1 expression in the brain, particularly in the rat hypothalamus. In these studies, we extend the anatomical localization to mouse brain and found high levels of Pgrmc1 mRNA in hypothalamic nuclei, yet the roles of Pgrmc1 in neural cells is not yet clear. At the cellular level, Pgrmc1 has been localized to the nucleus, suggesting a role in transcription. In order to investigate this possibility, we used whole-genome microarrays to examine the effects of Pgrmc1 levels on gene expression in a hypothalamic cell line. We report that Pgrmc1 co-regulates several gene targets, including nuclear receptor subfamily 4 group A member 1 (Nr4a1), interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), spectrin alpha 1 (Spna1) and transglutaminase 2 (Tgm2). Given the role of Pgrmc1 in signaling pathways of diverse tissues, we used pathway analysis software on our microarray data to determine if Pgrmc1 regulates signaling pathways in neural cells. We found that Pgrmc1 regulates basal levels of STAT3 activation in a manner that may explain Pgrmc1 coregulation of several target genes. Thus, we propose a novel role of Pgrmc1 in neural cells in regulation of STAT3 pathways and in regulating novel target genes.
5.2 Introduction

The observation that progesterone exerts rapid effects in diverse tissues has led the discovery of several novel signaling molecules. Among these molecules, Progesterone Receptor Membrane Component 1 (Pgrmc1) has been implicated in a wide array of physiological functions. For example, Pgrmc1 is required for the apoptotic actions of progesterone (P$_4$) in granulosa cells (Peluso et al., 2006, Peluso et al., 2008). In adrenal cells, Pgrmc1 mediates steroid biosynthesis and metabolism through interactions with enzymes in the biosynthetic pathway (Laird et al., 1988, Min et al., 2004, Hughes et al., 2007). Finally, in sperm cells, Pgrmc1 is implicated in the P$_4$-induced calcium influx that precedes the acrosome reaction (Buddhikot et al., 1999, Falkenstein et al., 1999). Together, these data indicate that Pgrmc1 may regulate divergent effects depending on the tissue type.

While Pgrmc1 has been most studied in non-neural tissues, high levels of Pgrmc1 have also been found in the brain, indicating that Pgrmc1 may play an important role in neural functions (Krebs et al., 2000, Intlekofer and Petersen, 2011). Pgrmc1 expression is also found in vasopressinergic neurons of the paraventricular nucleus (Meffre et al., 2005), a finding that may point to a role in water homeostasis. Though these studies yield clues as to the potential roles of Pgrmc1, the specific neural actions of Pgrmc1 have not been identified.

At the cellular level, evidence suggests that Pgrmc1 regulates diverse signaling pathways and transcriptional events, though the cellular actions of Pgrmc1 are likely to vary depending on the cell type. In breast cancer cells, Pgrmc1 activates Akt and IKB through phosphorylation (Hand and Craven, 2003) while in hippocampal neural
progenitor cells, Pgrmc1 is required for ERK pathway activation (Liu et al., 2009). Pgrmc1 may also alter transcription of target genes as supported by evidence that Pgrmc1 localizes to the nucleus (Beausoleil et al., 2004, Peluso et al., 2006) and may regulate cell proliferation and pro-survival genes (Liu et al., 2009, Peluso et al., 2010). Despite this wide range of clues, virtually nothing is known regarding Pgrmc1-dependent signaling pathways and gene targets in hypothalamic neural cells, where Pgrmc1 expression levels are especially abundant. In order to address this issue, we used a hypothalamic cell line and Pgrmc1 small interfering RNA (siRNA) to knock down Pgrmc1 expression, and identified novel gene candidates and Pgrmc1-dependent signaling pathways using microarray analyses.

5.3 Materials and Methods

5.3.1 Animals and Tissue Preparation

All animals were housed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and all protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts. Three adult female C57Bl/6 mice (21-22 g; Jackson Labs; Bar Harbor, ME) were communally housed in the Animal Care Facility with food and water provided *ad libitum* on a 14:10 light:dark cycle. Animals were ovariectomized under isofluorane anesthesia, allowed to recover for one week, and then injected s.c. with 10 µM 17β-estradiol dissolved in 100 µl sesame oil to ensure a similar hormonal milieu among animals. After 8 h, brains were collected and rapidly frozen on powdered dry ice and stored at -80°C until they were cryosectioned (Leica CM3000, Nussloch, Germany). 14-µm coronal sections through the region containing the hypothalamus were thaw-
mounted onto gelatin-coated slides and prehybridized as previously described (Ottem et al., 2004).

5.3.2 Cells and Culturing Methods

We used N42 cells, an immortalized hypothalamic neuronal cell line (Cellutions Biosystems, Inc.; Burlington, Ontario, Canada). Cells were maintained at 37°C and 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% (v/v) HyClone fetal bovine serum (FBS; Thermo Fisher Scientific, Rockford, IL), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (PS-Gln; Gibco-BRL; Gaithersburg, MA). Once seeded cells were reached approximately 70% confluence, they were rinsed with phosphate buffered saline (PBS) and media was replaced with phenol red-free DMEM with 10% charcoal-stripped FBS and PS-Gln. After 24 h, cells were transfected using siRNA or expression vectors as described below.

5.3.3 siRNA Experiments

N42 cells were transfected with 2 µM negative control or Pgrmc1 siRNA (Cat no. GS53328 and SI03650318, SABiosciences; Frederick, MD), using HiPerfect Transfection Reagent according to the manufacturer’s protocol (Qiagen, Valencia, CA). After 16 h, cells were treated with P₄ (10 µM) for an additional 8 h prior to harvesting RNA. The P₄ treatment did not alter Pgrmc1 levels (Figure 5.8.3a), a finding also shown in granulosa cells (Peluso et al., 2010). Total RNA was isolated using TRIzol reagent (Invitrogen; Carlsbad, CA) and Qiagen lipid easy kit (Qiagen) and manufacturer’s protocols.
5.3.4 Microarray Gene Expression Profiling

RNA from N42 cell siRNA experiments was used for Mouse Genome 430 2.0 Gene Chip assay (Affymetrix; Palo Alto, CA) performed by the Keck Microarray Institute at Yale University. RNA quality was assessed using an Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChips (Agilent Technologies, Palo Alto, CA). After data retrieval from the Keck Microarray Institute, Ingenuity Pathway Analysis and Pathway Studio (Ariadne; Rockville, MD) were employed to determine differences in gene expression that may differ among treatment groups. To determine possible signaling pathways differentially affected by Pgrmc1 siRNA treatments, I used Pathway Studio, which utilizes the ResNet database based on the assumption that functionally related genes are co-transcribed. I used Pathway Studio’s Gene Set Enrichment Analysis to identify candidate signaling pathways in which a higher number of pathway entities were significantly different between scramble and Pgrmc1 siRNA-transfected samples.

5.3.5 Pgrmc1 Construct

N42 cells were transiently transfected using a pcDNA3.3 TOPO TA vector (Invitrogen; Carlsbad, CA) containing Pgrmc1 and using manufacturer’s protocol. To generate the Pgrmc1 product, primers were obtained from Integrated DNA Technologies (Coralville, IA) and PCR reagents (Promega; Madison, WI). The coding portion of the mouse Pgrmc1 sequence was produced using PCR primers: 5’- TCATGGCTGCCGAGGATGT-3’ and 5’-TTATACATTCATTCTTCCGAGC-3’ corresponding to sequence 61-650 and included a stop signal (as denoted by underline). Gel-purified PCR products were cloned into the pcDNA™ 3.3-Topo® TA Cloning Kit.
using manufacturer’s protocol (Invitrogen). Transformants were analyzed for incorporation and orientation of the product using primers provided by manufacturer and sequencing. I linearized the plasmid construct using Pvu I prior to transfection into N42 cells, and purified the linearized construct by phenol-chloroform extraction and ethanol precipitation. The empty vector or Pgrmc1 expression construct was transfected into N42 cells using SuperFect and manufacturer’s protocol (Qiagen) and the resulting increase in Pgrmc1 RNA and protein was confirmed by QPCR and western blot (Figure 5.8.6).

5.3.6 Dual-luciferase Assays

The transcriptional activity of STAT3 was measured using dual-luciferase assays following transfection of N42 cells with Cignal™ STAT3 reporter construct (Cat. no CCS-9028L; SABiosciences). In addition to STAT3-responsive elements upstream of the firefly luciferase construct, a Renilla luciferase construct was included in the same construct as an internal control for transfection efficiency normalization. To optimize transfection conditions, positive and negative control plasmids and manufacturer’s protocol were used (SABiosciences). Combined transfection with siRNA was performed in order to determine the effect of Pgrmc1 knockdown on STAT3 activity in N42 cells. Renilla and firefly luciferase activities in cell lysates were assayed using the Dual Luciferase reporter system (Promega) and measured on a Fluostar Optima Luminometer (BMG; Durham, NC). Experiments were performed in triplicate and results are presented in Figure 5.8.5a.
5.3.7 Quantitative Reverse Transcriptase PCR (QPCR)

QPCR was used to verify that Pgrmc1 was knocked down by treatment with Pgrmc1 siRNA compared to scramble siRNA controls, and in order to validate outcomes of our microarray study. Genes were chosen for validation based on their high and low levels of expression in N42 cells treated with scramble or Pgrmc1 siRNA in the presence of P₄. N42 RNA samples were generated using the identical treatment paradigm as described above for the original microarray experiment. RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcriptase Kit (Roche Diagnostics, Indianapolis, IN) and manufacturer’s protocol, which included a genomic DNA degradation step. Primers were obtained from SABiosciences for nuclear receptor subfamily 4 group A member 1 (Nr4a1; 24496A), interferon-induced protein with tetratricopeptide repeats 3 (IFIT3; 06008A), transglutaminase 2 (Tgm2; 40872E) and spectrin alpha 1 (Spna1; 32969A) and manufacturer’s protocol. Primers for Pgrmc1 and β-actin were obtained from Integrated DNA Technologies (Coralville, IA) and are listed in Table 5.7.1. Reactions contained reagents from QuantiTect SYBR Green Kit, following manufacturer’s protocol (Roche Diagnostics, Indianapolis, IN). These reactions were carried out using a Stratagene MX3000P thermocycler (Agilent Technologies, Wilmington, DE) and the following parameters: 95°C for 10 min, and 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. MxPro™ QPCR analysis software (Agilent Technologies) was used to obtain fluorescence measurements. Threshold cycle readings were used to calculate the relative amounts of target genes by normalizing them to the signal of β-actin after validating that β-actin mRNA levels did not differ among treatments. Primer specificity was also validated
using 2% agarose gel electrophoresis to verify single products following the QPCR reaction.

5.3.8 Western Blot

Protein was isolated from N42 cells lysates using radioimmunoprecipitation assay buffer (Boston BioProducts, Worcester, MA) containing 1% protease inhibitor cocktail (Calbiochem, San Diego, CA) and manufacturer’s protocol. Protein concentrations were assessed using Pierce bicinchoninic acid protein assay (Thermo Fisher Scientific). Ten-µg of protein per lane was electrophoresed on 4–15% Tris–HCl SDS-PAGE gradient gels (Biorad; Hercules, CA) and was transferred electrophoretically to Immobilon-P membranes (Millipore; Billerica, MA.) Membranes were blocked in 5% skimmed dry milk in Tris-buffered saline plus 0.05% Tween-20 (TBST; pH 7.4) for 1 h at room temperature on an orbital shaker, and then incubated overnight at 4°C with anti-Pgrmc1 antibody (1:1000; Proteintech Group, Chicago, IL) in TBST. Membranes were washed in TBST for 30 min, followed by horseradish peroxidase-conjugated secondary antibody (anti-rabbit 1:10,000; Abcam, Cambridge, MA) for 1 h, and developed using Chemiluminescence HRP Substrate (Millipore). Blots were apposed to BioMax MR film (Kodak; Rochester, NY), and band intensities were quantified from developed films using densitometric software (GeneTools ver. 3.07 SynGene; Cambridge, England). Blots were re-probed with anti-β-actin (1:2000; Abcam) and anti-mouse antisera (1: 2000; Abcam) to provide protein loading controls.
5.3.9 Janus Kinase (JAK) and Signal Transducer and Activator of Transcription 3 (STAT3) Inhibition

To determine whether activation of Jak2/STAT3 signaling pathway was upstream of Pgrmc1 siRNA effects on gene targets, N42 cells were treated with DMSO vehicle control or 10 nM JSI-124 (cat. no. C4493; Sigma). JSI-124 is a cell-permeable compound that specifically inhibits Jak2 and STAT3 by suppressing their phosphorylation status (Sun et al., 2005, Shi et al., 2006). JSI-124 exposure decreases STAT3 DNA binding and STAT3-mediated gene expression, yet has no effect on the activity of Src, Akt, ERK, or JNK pathways (Blaskovich et al., 2003). The dose of 10 nM was selected based on preliminary reports of JSI-124 effective doses in other cell lines, and a dose-response of N42 cell viability following 24 h exposure to vehicle, 1x10^{-6} M, 1x10^{-5} M, 1x10^{-4} M and 1x10^{-3} M JSI-124. A trypan blue exclusion assay was used to assess cell viability as previously described (Mishell and Shiigi, 1980) and cell phenotype changes were also recorded (Figure 5.8.4a).

5.3.10 Oligodeoxynucleotidyl Probe Preparation

Sense and antisense oligodeoxynucleotidyl probes were obtained from Integrated DNA Technologies (Coralville, IA) as listed in Table 1. Using terminal deoxynucleotidyl transferase (Roche, Indianapolis, IN), sequences were 3’-end labeled with \([\alpha^{33}P]dATP\) (PerkinElmer, Waltham, MA) as described previously (Petersen et al., 1989). I purified the probe by phenol-chloroform extraction and ethanol precipitation and washed the pellet with 70% ethanol before suspending the probe in 25 µl TE (10 mM Tris-HCl; pH 8.0, 1 mM EDTA).
5.3.11 In Situ Hybridization Histochemistry (ISHH)

To determine the relative localization of Pgrmc1 and validated gene targets, radioisotopic probes (0.5×10^6 cpm) were applied to prehybridized brain sections in 20 µl hybridization buffer. This buffer contained 4×SSC (1×SSC=0.15 M NaCl/0.015 M sodium citrate, pH 7.2), 50% (v/v) formamide, 10% (w/v) dextran sulfate, 250 µg/ml yeast tRNA, 1× Denhardt's solution, 500 µg/ml heparin sodium salt, 0.1% sodium pyrophosphate and 0.05 M dithiothreitol added freshly before use. After covering sections with glass coverslips, slides were hybridized for 16 h at 37 °C in humidified plastic boxes. Slides were removed from 37 °C and coverslips were floated off in 1×SSC. Sections were washed four times for 15 min each in 2×SSC-50% formamide solution at 40 °C, followed by four washes, 15 min each, in 1×SSC. All sections were rinsed in double-distilled water, dehydrated briefly in 70% ethanol, and air-dried. For signal detection, slides were apposed to BioMax MR film (Kodak) and autoradiograms were developed by an X-ray film processor. I then used NTB2 emulsion and standard autoradiographical procedures to visualize probes for Nr4a1 (Kodak; 10 d exposure) and counterstained brain sections using thionin in order to determine whether Nr4a1 signal was confined to cell bodies. All images were acquired using BioQuant Imaging Software (Bio-Quant Inc., Nashville, TN, USA) and a CCD videocamera (QImaging QICAM FAST color, Surrey, BC, Canada).

5.3.12 Statistics

Data is expressed as means ± SEM and all data shown is the result of at least three replicates of three or more independent runs for each assay. Treatment effects were evaluated using one-way ANOVA, except for validation of QPCR studies using
siRNA and P₄ treatments which required two-way ANOVA. Bonferroni t-tests were used for post hoc analyses of treatment effects. For QPCR studies, Ct values from duplicates of each sample were normalized to those of β-actin and analyzed using the ∆∆Ct method (Livak and Schmittgen, 2001) and treatment effects were evaluated using two-way ANOVA. Dual luciferase experiments were analyzed by unpaired t tests.

5.4 Results

5.4.1 Pgrmc1 siRNA Experiments

Pgrmc1 expression was depleted by treatment with 2 µM Pgrmc1 siRNA compared to scramble siRNA controls as determined by QPCR (Fig. 5.8.1a) and western blotting (Fig. 5.8.b). Knockdown of Pgrmc1 was significant at 16 h post-transfection with siRNA, and persisted at 24 h (Fig 5.8.1a). Following optimization of the siRNA transfection protocol, RNA was prepared for microarray analyses and validation studies.

5.4.2 Identification of Novel Target Genes by Microarray Analyses

Genes identified using Affymetrix whole-genome microarrays are displayed using a heat map and fold change analyses (Fig. 5.8.2a). To generate fold changes between scramble (control) and Pgrmc1 siRNA-transfected groups, the mean expression values of samples transfected with Pgrmc1 siRNA were log₂ transformed and normalized to those of the control group. The raw expression values of gene expression among groups were used to run gene set enrichment analysis in order to provide a ranking of predicted canonical pathways that differ when Pgrmc1 expression was decreased by the use of Pgrmc1 siRNA (Table 5.7.3). The top-ranked pathway was IL6R and STAT3 signaling and is displayed in Figure 5.8.2b with relative expression.
data between scramble (control) and Pgrmc1 siRNA-transfected groups as indicated (Fig. 5.8.2a).

5.4.3 Validation of Microarray Findings by QPCR

Several gene candidates differentially expressed following Pgrmc1 depletion were validated by QPCR in N42 cells. In these experiments, N42 cells were also exposed to 8 h of 10 nM P₄, and levels of Pgrmc1 mRNA were validated to be decreased by transfection with Pgrmc1 siRNA compared to scramble (controls) (Fig.5.8.3a). Pgrmc1 depletion decreased levels of mRNA encoding pro-survival genes Tgm2 (Fig.5.8.3b) and Spna1 (Fig.5.8.3.c). Additional gene candidates were selected and validated to be increased by Pgrmc1 depletion, including genes implicated in apoptotic pathways including Nr4a1 (Fig. 5.8.3d) and IFIT3 (Fig. 5.8.3.e). Regulation of Pgrmc1 and all gene targets was P₄-independent at the dose tested.

5.4.4 Transfection of Pgrmc1 Expression Construct

To determine if Pgrmc1 levels resulted in the changes to mRNA levels as shown in siRNA depletion studies, Pgrmc1 expression constructs were used to increase Pgrmc1 expression in N42 cells. Transfection of empty vector control or Pgrmc1 expression constructs into N42 cells increased levels of Pgrmc1 mRNA (Fig. 5.8.4a) and protein (Fig. 5.8.4b). In these experiments, Pgrmc1 over-expression causes similar increases in levels of mRNA encoding Tgm2 (Fig. 5.8.4c) and Spna1 (Fig. 5.8.4d). Over-expression of Pgrmc1 appeared to decrease Nr4a1 levels (Fig. 5.8.4.e), and had no effect on IFIT3 mRNA (Fig 5.8.4.f).
5.4.5 Pgrmc1 Levels Regulate STAT3 Activity

In accordance with pathway candidates identified by microarray analyses, the relative activity of STAT3 was investigated using a STAT3 reporter construct. Dual-luciferase assays were performed on N42 cells cotransfected with a STAT3 reporter and scramble (control) or Pgrmc1 siRNAs. After 24 h of transfection, cells treated with Pgrmc1 siRNA showed increased STAT3 activation relative to scramble siRNA controls, (Fig. 5.8.5).

5.4.6 STAT3 Inhibitor JSI-124 Alters Pgrmc1, Nr4a1 and IFIT3 mRNA Levels

Treatment of N42 cells with doses of JSI-124 resulted in rounding of the cells and cell death as determined by trypan blue exclusion assay (Fig. 5.8.6a), therefore a dose of 10 nM was chosen as to minimize changes in cell morphology and viability. Treatment of N42 cells with JSI-124 for 8 h decreased Pgrmc1 mRNA levels compared to vehicle controls (Fig. 5.8.6a). This treatment had no effect on levels of mRNA encoding Tgm2 (Fig. 5.8.6c) or Spna1 (Fig. 5.8.6d), but increased Nr4a1 mRNA levels (Fig. 5.8.6e) and decreased IFIT3 mRNA levels (Fig. 5.8.6f).

5.4.7 ISHH Studies Reveal That Pgrmc1, STAT3, and Nr4a1 Localize to the Hippocampus and Paraventricular Nucleus of Mouse Brain

ISHH studies were performed in order to determine the anatomical localization of Pgrmc1 and whether Pgrmc1-coregulated genes identified in N42 cells were present in the same brain regions. Pgrmc1 signal was especially abundant in neuroendocrine nuclei including the medial preoptic area (Fig. 5.8.7g), suprachiasmatic nucleus (Fig. 5.8.7j), paraventricular nucleus (Fig. 5.8.7m) and hippocampus (Fig. 5.8.7p). Nr4a1 signal was high in the cortex and rostral striatum (Fig. 5.8.7 e, h, k), and very strong in
the paraventricular nucleus (Fig. 5.8.7n) and hippocampus (Fig. 5.8.6q). STAT3 ISHH signal was very diffuse but appeared to overlap the expression of Nr4a1 and Pgrmc1 in the paraventricular nucleus (Fig. 5.8.7o) and hippocampus (Fig. 5.8.7r). ISHH signal for STAT3 and Nr4a1 in the paraventricular nucleus have been reported previously (Parkes et al., 1993, Hubschle et al., 2001). The specificity of the Nr4a1 ISHH signal in the paraventricular nucleus was further confirmed by autoradiographical detection using emulsion as shown in Figure 5.8.8a. Specific ISHH signal IFIT3, Spna1, and Tgm2 was not detected (data not shown).

5.5 Discussion

We found that Pgrmc1 levels regulate basal levels of mRNA encoding several novel gene targets in neural cells, including Spna1, Tgm2, Nr4a1 and IFIT3, indicating a novel role of Pgrmc1 in regulating diverse genes in neural cells. While mRNA levels of Tgm2 and Spna1 closely matched those of Pgrmc1, levels of Nr4a1 and IFIT3 were increased following Pgrmc1 depletion, suggesting that Pgrmc1 exerts repressive effects on gene expression. Our novel findings that Pgrmc1 regulates STAT3 activation indicate that Pgrmc1 levels may regulate basal activation of pathways critical for cell proliferation. Finally, the results of our ISHH studies show that Pgrmc1, Nr4a1 and STAT3 mRNA signal is enriched in the hippocampus and paraventricular nucleus, in line with the idea that Pgrmc1 levels may regulate Nr4a1 abundance and STAT3 activation in vivo. Interestingly, the effects of Pgrmc1 depletion on Tgm2, Spna1, Nr4a1 and IFIT3 were P4-independent, suggesting that Pgrmc1 exerts important functions at the cellular level even in the absence of P4. Taken together, our findings provide a novel role of Pgrmc1 in regulating basal levels of novel gene targets and
STAT3 signaling, and indicate that Pgrmc1 may function as a pro-survival factor that regulates cell maintenance and proliferation.

Our results demonstrate that Pgrmc1 levels regulate levels of several genes involved in cell cycle progression and proliferation, which may provide insight into the role of this molecule in mitotic neurons rather than in adults. While this was not the original intention of the study, the validated gene targets are relevant to pro-survival functions in developing neural cells. For example, Pgrmc1 repression decreased levels of Spna1, a gene known for maintenance of cytoskeletal structures and for the regulation of plasma membrane components (Beck and Nelson, 1996). Spna1 is a molecule found in all major cell types of the brain and interacts with proteins important for synaptic function (Krebs et al., 1987, Sikorski et al., 1991, Clark et al., 1994). This ubiquitous expression in brain cells is a possible reason for why specific ISHH Spna1 signal was not detected in this study, but as the genes identified point to roles in cell maintenance and proliferation, it is possible that the effect of Pgrmc1 on Spna1 levels is more pertinent to studies in the developing nervous system.

In addition to coregulation of Spna1, we found that Pgrmc1 levels appeared to dictate Tgm2 mRNA abundance, which is interesting because Tgm2 is heavily implicated in cell survival. Tgm2 levels are carefully controlled as this multifunctional enzyme regulates cell viability by controlling protein scaffold cross-linking and stabilizing integrin (Lorand and Graham, 2003, Janiak et al., 2006). Although we and others did not detect specific localization of Tgm2 mRNA by ISHH mouse brain (data not shown), Tgm2 is present in the brain as identified by QPCR and enzymatic activity assays (Gilad and Varon, 1985, Bailey et al., 2004). Much like Pgrmc1, Tgm2
expression is sharply increased by traumatic brain injury (Gilad et al., 1985, Tolentino et al., 2002, Labombarda et al., 2003, Guennoun et al., 2008), indicating that these genes may be required for recovery processes. Importantly, Tgm2 is also important for neural development, in line with the idea that it may play important roles in actively-dividing cells (Spiteri et al., 2007).

We found that Pgrmc1 depletion increased levels of Nr4a1 and IFIT3 mRNA, which may indicate a repressive role of Pgrmc1 on transcription of these genes and support the notion that Pgrmc1 is important for cell viability. Both Nr4a1 and IFIT3 mediate apoptosis (Chan et al., 1998a, Chintharlapalli et al., 2005, Stawowczyk et al., 2010); therefore, Pgrmc1 repressive effects on their expression may represent a pro-survival function. These genes have distinct functions in other cells that may yield clues as to their neural roles. Pgrmc1 regulation of Nr4a1 is especially interesting as both genes regulate aspects of P₄ metabolism, though they have not been previously linked. Specifically, Nr4a1 is a member of the nuclear receptor superfamily that acts as a transcription factor and induces expression of 20-α-HSD, an enzyme that decreases progesterone levels. While a steroid metabolism role is suggested by studies on Nr4a1 in adult cells, in mitotic cells, Nr4a1 is specifically expressed during the G0/G1 cell cycle re-entry phase (Hazel et al., 1988). Although less is known regarding the function of IFIT3, it is induced by immunogenic stimuli and is STAT-dependent (Wang and Campbell, 2005), in line with our finding that STAT3 inhibition decreases IFIT3 mRNA levels. IFIT3 expression is difficult to localize to the brain without immunogenic stimuli (Wacher et al., 2007), a possible reason for why specific IFIT3 RNA signal was not detectable in our ISHH studies (data not shown). Thus, Pgrmc1’s
repressive effect on levels of Nr4a1 and IFIT3 are consistent with pro-survival functions in neural cells.

Our finding that Pgrmc1 regulates STAT3 signaling also supports a role of Pgrmc1 in cell cycle progression, but this conclusion is likely dependent on cell-type. Studies using cell lines show that STAT3 pathway activation is important for cell cycle progression, and this pathway is also important in mediating immune responses, differentiation and apoptosis (Nakajima et al., 1996, Fukada et al., 1998). By inhibiting STAT activation using JSI-124, we were able to see a decrease in mRNAs encoding Pgrmc1 and IFIT3. In the case of IFIT3, this was a predicted result as IFIT3 has been shown to be increased coordinately with JAK/STAT signaling (Darnell, 1998). The observed increase in Nr4a1 may be a compensatory rise in response to declining Pgrmc1 levels. The observed decreases in levels of Pgrmc1 mRNA suggest that Pgrmc1 levels and STAT signaling are coordinately regulated and useful indicators of cell proliferation in vitro. Indeed, this idea may be more relevant for actively-dividing cells rather than for post-mitotic neurons.

Although STAT3 signaling correlates to cell proliferation, STAT3 signaling is integral to a host of physiological effects depending on the tissue. Importantly, neural STAT3 signaling is important for mediating leptin actions in hypothalamic nuclei to control body weight (Hubschle et al., 2001). Thus, our findings of high Pgrmc1 and STAT3 ISHH signal in the paraventricular nucleus may reflect a role in regulating basal responses to leptin. The overlap of these with Nr4a1 expression is also interesting as Nr4a1 is induced by stress. Taken together, the role of Pgrmc1 appears to be important
for restraining STAT activity, and may be relevant to STAT signaling in the brain, though this possibility must be tempered by future research.

In conclusion, neural expression of Pgrmc1 may be relevant for a host of cell processes, including regulation of STAT pathway activation and the expression of several gene targets. Indeed, our present work contributes to a growing body of evidence that STAT3 pathways are tightly regulated by many diverse cellular factors. Extensive work in the field of JAK/STAT signaling shows that STAT3 signaling is also constrained by a large family of diverse proteins (Lufei et al., 2003; Zhange et al., 2003; Wormald and Hilton, 2004). Our findings suggest novel cross-talk signaling between Pgrmc1 and STAT3 signaling and suggest that novel gene targets, including Spna1, Tgm2, Nr4a1, and IFIT3, may be important Pgrmc1 targets in mitotic neurons. Given the relevance of STAT signaling in the paraventricular nucleus, a brain region in which we identified high levels of Pgrmc1 and Nr4a1, a potential role of Pgrmc1 may also be implicated involving stress and body weight homeostasis.
### 5.6.1: Primers Used in QPCR Studies

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5.6.2: Sequences for Oligodeoxynucleotidyl Probes Used in ISHH Studies

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## 5.6.3: Gene Set Enrichment Analysis

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<td>5</td>
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5.7.1 Pgrmc1 siRNA Decreased Levels of Pgrmc1 mRNA and Protein

Figure 5.7.1: Pgrmc1 siRNA Decreased Levels of Pgrmc1 mRNA and Protein. N42 cells transfected with either scramble (control) or Pgrmc1 siRNA had decreased levels of Pgrmc1 mRNA at 16 and 24 h post-transfection as determined by QRTPCR (A). Treatment with Pgrmc1 siRNA for 24 h decreased Pgrmc1 protein as determined by western blot (B) to approximately 50% that of scramble siRNA controls (C). ** $p \leq 0.01$. 
5.7.2 Analyses of Microarray Data

Figure 5.7.2: Analyses of Microarray Data. Heat map representation of a subset of differentially expressed genes in N42 cells following transfection with scramble (control) or Pgrmc1 siRNA (A). Gene Set Enrichment Analysis identified the IL6R and STAT signaling pathway as a candidate pathway affected by Pgrmc1 siRNA transfection relative to scramble (control) (B). Shading of pathway entities indicates the relative gene expression of scramble (control) versus Pgrmc1 siRNA-transfected N42 cells. As illustrated, Pgrmc1 siRNA modestly increased all pathway members relative to scramble siRNA.
5.7.3 Validation of Microarray Findings by QPCR

Figure 5.7.3: Pgrmc1 co-regulates multiple gene targets in a P₄-independent manner. N42 cells transfected with scramble (control) or Pgrmc1 siRNA were treated with vehicle control (Veh) or 10 nM P₄. Pgrmc1 siRNA transfection depleted Pgrmc1 mRNA levels to 50% that of controls (A). Pgrmc1 co-regulated mRNA levels of Tgm2 (B) and Spna1 (C). Pgrmc1 depletion increased levels of Nr4a1 (D) and IFIT3 (E) in a P₄-independent manner. *** = p ≤ 0.001
5.7.4 Pgrmc1 Construct Transfection Increased Pgrmc1 Expression

Figure 5.7.4: QPCR was used to determine relative levels of mRNA in N42 cells transfected with empty vector (control) or Pgrmc1 construct. Pgrmc1 mRNA (A) and protein (B) levels were increased by transfection with a Pgrmc1 construct compared to controls. $* = p \leq 0.05$. Pgrmc1 co-regulated mRNA levels of Tgm2 (C) and Spna1 (D). Pgrmc1 overexpression decreased levels of Nr4a1 (E) but had no effect on levels of mRNA encoding Ifit3 (F). $* = p \leq 0.05$; $** = p \leq 0.01$; $*** = p \leq 0.001$. 
5.7.5 Pgrmc1 Depletion Stimulates STAT3 Activation

Figure 5.7.5: Depletion of Pgrmc1 increased STAT3 activation. (A) N42 cells were cotransfected with a STAT3-luciferase reporter and either scramble (white bar) or Pgrmc1 (black bar) siRNA for 24 h prior to assaying luciferase activities. The STAT3 construct contained multiple STAT3-responsive elements upstream of luciferase and an internal Renilla reporter for normalization, and relative luciferase activity of cell lysates is shown. ** = p ≤ 0.01.
5.7.6 Inhibition of JAK/STAT in N42 Cells

Figure 5.7.6: Effects of JAK/STAT Inhibition for 8 h. N42 cells exposed to varying doses of JSI-124 exhibit cell rounding and cell death as determined by phenotypic and trypan blue exclusion assays (A). Exposure to 10 nM JSI-124 for 8 h reduced levels of Pgrmc1 mRNA (B), had no effect on levels of mRNA encoding Tgm2 (C) or Spna1 (D), modestly increased Nr4a1 mRNA levels (E), and reduced IFIT3 mRNA levels (F). * = $p \leq .05$; ** = $p \leq .01$; *** = $p \leq .001$. 


5.7.7 Localization of Pgrmc1, Nr4a1 and STAT3 mRNA in Mouse Brain

Figure 5.7.7: Film autoradiograms of sections hybridized to $^{33}$P-labeled oligodeoxynucleotidyl probes for Pgrmc1, Nr4a1 and STAT3. Representative sections hybridized to sense strand probes are shown in the row one (A, B and C), and antisense probes were used all other sections shown. Black arrows show high signal intensities in the paraventricular nucleus for Pgrmc1 (M), Nr4a1 (N) and STAT3 (O) and the hippocampus for Pgrmc1 (P), Nr4a1 (Q) and STAT3 (R). Scale bar = 350 µm.
5.7.8 Cell-specific Nr4a1 ISHH Signal in the Paraventricular Nucleus

Figure 5.7.8: Cell-specific Nr4a1 ISHH Signal in the Paraventricular Nucleus. Nr4a1 ISHH signal is highly specific for cells of the paraventricular nucleus as indicated by silver grains ($^{33}$P-labeled oligodeoxynucleotidyl probes antisense for Nr4a1). The black arrows indicate silver grain signal. Scale bar = 30 µm; 3V = Third ventricle.
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CHAPTER 6

CONCLUDING REMARKS

6.1 Main Findings Concerning the Neural Roles of Pgrmc1

The series of studies presented herein provide significant new insight into the neural roles of Pgrmc1. According to my working premise, I have identified factors that implicate Pgrmc1 in the context of neural function. As a molecule primarily studied in ovarian cells, Pgrmc1 has now emerged as a P$_4$ candidate signaling molecule in the brain. As the first to map and compare the anatomical distributions of Pgrmc1, Pgrmc2, Serbp1, Paqr7 and Paqr8, my findings are consistent with the idea that these signaling molecules may regulate neuroendocrine functions. Moreover, these studies strengthened the concept that Pgrmc1 interacts with Serbp1 in many brain nuclei as they were shown to display strikingly similar distributions through much of the forebrain. Furthermore, based on the overlapping distributions of the classical Pgr with Pgrmc1, my work provides the first indication that P$_4$ may influence neural functions through multiple signaling molecules.

In further support of reproductive roles of Pgrmc1, I examined the regulation of mRNA levels by ovarian hormones and found that these are regulated in a nuclei-specific manner. As these specific neuroendocrine brain regions control diverse reproductive functions, the finding that Pgrmc1 expression responded to ovarian hormones depending on brain region is consistent with its ability to mediate diverse responses depending on cell type. In further support of important neural functions, the pattern of regulation of binding partners Pgrmc1 and Serbp1 was nearly identical in several nuclei examined, suggesting that they may work together to mediate P$_4$ effects.
Comparisons of gene expression patterns were facilitated by examining the regulation of Paqr7, Paqr8, the classical estrogen receptor α (Esr1) and Pgr. My finding that patterns of regulation for Pgrmc1 and Pgr overlap in some regions suggests that these P₄ signaling molecules may act together, but their overlapping distribution were not complete. The presence of Pgrmc1 in Pgr-deficient hypothalamic nuclei may point to an independent role of Pgrmc1 in the neuroendocrine effects of P₄.

Key findings from our studies using dual-label in situ hybridization are the first to show that GABAergic AVPV neurons contain abundant Pgrmc1 and Serbp1. Given the indispensable role of these neurons in the preovulatory LH surge, these findings indicate that Pgrmc1 may be important in the neural control of ovulation. In addition to their presence in GABAergic neurons, Pgrmc1 was specifically found within Serbp1-containing neurons, which emphasizes the likelihood that these molecules function together as part of a P₄–receptor complex as demonstrated in other cell types.

The cellular roles of Pgrmc1 were investigated using a hypothalamic neural cell line, and my results demonstrated that Pgrmc1 levels dictate the expression of several genes. By validating microarray findings, I found that Pgrmc1 coregulated several novel genes targets, including Tgm2 and Spna1. Given that these genes have important anti-apoptotic actions, it is likely that regulation of these genes is important for cell viability. In light of these data, I propose a critical action of Pgrmc1 in mediating pro-survival functions through transcriptional regulation of levels of several target genes. In further support of this notion, Pgrmc1 levels also had a reciprocal effect on levels of mRNA encoding Nr4a1 and IFIT3, gene targets involved in apoptotic signaling (Liu et al., 1994, Guenterberg et al., 2010). In further pro-survival functions, I found evidence
that Pgrmc1 affects basal STAT3 activation levels. Interestingly, STAT inhibition decreased Pgrmc1 levels as well, suggesting cross-talk between Pgrmc1- and STAT3-dependent pathways.

Interestingly, levels of Pgrmc1-coexpressed genes were not sensitive to P₄ in our hypothalamic cell line, demonstrating that Pgrmc1 exerts P₄-independent effects on gene expression. While it is possible that Pgrmc1 exerts these important effects in differentiated neurons, instead Pgrmc1 actions may be different in mitotic cells such as our hypothalamic cell line. Although we found that the paraventricular nucleus contains high levels of Pgrmc1 and specific expression of STAT3 and Nr4a1, the role of the gene targets identified suggests that Pgrmc1 may affect cell viability. Furthermore, this idea could be more relevant in mitotic cells during development than in differentiated adult neurons of the hypothalamus.

These novel insights into Pgrmc1 function expand the potential roles of this signaling molecule, and have raised important possibilities as to Pgrmc1 roles in nuclei that rely upon STAT3 activation for neurobehavioral outcomes. Moreover, by identifying novel gene targets sensitive to Pgrmc1 levels in a neural cell line, this work indicates a P₄-independent role of Pgrmc1 that may impact the developing brain. Taken together, the work presented here links Pgrmc1 to cell signaling cascades and gene targets important for neural function, suggesting that Pgrmc1 may be important for a broad array of pro-survival actions in neurons.

In further support of Pgrmc1 as a pro-survival gene, Pgrmc1 levels are overexpressed by cancerous cells (Rohe et al., 2009, Peluso et al., 2008). In fact, Pgrmc1 levels have been proposed to promote tumorigenesis (Ahmed et al., 2010) and
increases basal activation of Iκb (Hand and Craven, 2003), an inhibitor of NFκB signaling that regulates cell viability (Baeuerle and Baltimore, 1988). While further studies are warranted to determine the specific cellular mechanisms, Pgrmc1 appears to promote survival in mitotic cells (Peluso et al., 2008). This idea is also supported by my findings that Pgrmc1 regulates novel genes previously found to impact cell viability, including Tgm2, Spna1, Nr4a1 and IFIT3. An illustration including my findings concerning the cellular roles of Pgrmc1 is provided in Figure 6.2.1.

6.1.1 Pgrmc1 Roles in N42 Cells

![Diagram of Pgrmc1 Roles in N42 Cells]

Figure 6.1.1: Results of studies using N42 cells, a hypothalamic cell line, indicate that Pgrmc1 regulates levels of mRNA encoding Tgm2, Spna1, Nr4a1 and IFIT3. Pgrmc1 inhibited STAT3 activity as determined by experiments using a STAT3 reporter. JSI-124 is a STAT3 inhibitor that prevents phosphorylation of STAT3 pathway members. Treatment of N42 cells with JSI-124 resulted in decreased levels of mRNA encoding Pgrmc1 and IFIT3 (a STAT3-dependent gene). Taken together, these findings suggest potential cross-talk between Pgrmc1 and STAT3 signaling pathways that may culminate in alterations to gene expression.
6.2 Future Directions

While the anatomical localization studies and AVPV GABAergic expression of Pgrmc1 strongly imply a role in reproduction, an understanding of Pgrmc1 function will be greatly aided by the use of specific antagonists. A putative Pgrmc1 ligand known as AG-205 has been identified using the Arabidopsis thaliana homolog of Pgrmc1 (Yoshitani et al., 2005). This aromatic compound was used in further studies in breast cancer cells, and it appears to bind Pgrmc1 and inhibit cell viability and cell cycle progression in a Pgrmc1-dependent manner (Ahmed et al., 2010). While the specificity of AG-205 to Pgrmc1 requires further validation, this molecule may be used in future studies to investigate the neural role of Pgrmc1.

Additional work using transgenic animals will greatly aid our understanding of Pgrmc1 function. A role in important aspects of neural development has been suggested by several studies (Sakamoto et al., 2004, Runko et al., 1999), so the use of tissue-specific conditional knockouts may be better suited to investigate the role of Pgrmc1 in specific neural subpopulations. Despite our findings that GABAergic AVPV neurons express Pgrmc1, this subpopulation is a difficult target due to ubiquitous expression of GABA in many brain regions; therefore, a future possibility may be to target kisspeptin neurons. These neurons are found as a very discrete population in the medial AVPV along the third ventricle, and are critical for the LH surge mechanism in the mouse (Gottsch et al., 2004). Another possibility may be a Pgrmc1 knockout within neurons expressing steroidogenic factor-1, which would deplete Pgrmc1 expression primarily within neurons of the ventromedial nucleus. This idea may be another possibility worthy of future studies, particularly if feminine sex behaviors were
examined. Taken together, the characterization, regulation, and specific expression of Pgrmc1 in neurons important for control of reproduction provides an exciting opportunity for future studies using transgenic mice.

6.3 Closing Remarks

While the neural role of Pgrmc1 remains unclear, the full spectrum of research on this molecule reflects diverse implications of Pgrmc1 function. Based on its expression in a wide range of tissues, Pgrmc1 is likely to be important for a variety of physiological processes. In virtually all cell lines tested, Pgrmc1 exerts pro-survival actions, suggesting that this molecule is required for cell proliferation and viability. As a highly expressed gene in terminally differentiated neurons, Pgrmc1 may exert additional effect such as mediating P4 signaling or metabolism. The present work also suggests that Pgrmc1 levels may modulate signaling cascades in adult neurons. Thus, the results provided advance our understanding of Pgrmc1 in the context of neural function and yield new potential avenues of study for this molecule.


