Differentially expressed PR-A and PR-B are co-localized with SRC-1 and SRC-2 in the female mouse hypothalamus

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Abstract

The ovarian steroid hormones estradiol (E) and progesterone (P) act in the brain to influence reproductive behavior through binding to their respective receptors, estrogen receptors (ER) and progestin receptors (PR). Once bound to E, ER induces the expression of PR. There are two major isoforms of progestin receptors (PR): full length PR-B and the N-terminally truncated PR-A. Previous studies have shown that steroid receptor coactivator-1 (SRC-1) and SRC-2 are known to be expressed in estradiol-induced PR cells in the hypothalamus of female rodents, which provides anatomical evidence for their involvement in E-induced PR expression. Previous studies have not examined the coexpression of either PR-A or PR-B with SRCs. We hypothesize that: (1) PR-A and PR-B are differentially expressed in the hypothalamus, (2) SRC-1 and SRC-2 are differentially expressed with PR-A and PR-B, and (3) The presence of SRC-1 or SRC-2 correlates with increased PR expression in a cell. To provide evidence for these hypotheses, we used female transgenic mice that have been engineered to express either PR-A (PRBKO mice) or PR-B (PRAKO mice). Triple label immunohistochemistry was used to determine the localization of PR, SRC-1, and SRC-2 in regions of the brain involved in female sexual behavior including the ventromedial nucleus (VMN), the arcuate nucleus (ARC), and the medial preoptic area (MPA). We found that E differentially induces PR-A and PR-B in the hypothalamus. In the VMN, both PR-A and PR-B are E-induced, while in the ARC and MPA only PR-A is E-induced. Furthermore there are more PR-A cells than PR-B cells in the hypothalamus. When PR intensity was examined, we found that PR cells in wild type (WT) mice were more intense than PR-B cells, which suggests that there is less PR-B expression within cells of PRAKO mice. We also found that the majority of PR cells also express SRC-1 and SRC-2. In the VMN of WT mice, PR cells that also express SRC-2 are more intense than other PR cells. In the MPA of WT mice, PR cells that also express both SRC-1 and SRC-2 are more intense than other PR cells. Our findings show the novel differential induction of PR-A and PR-B in the hypothalamus of female mice. Additionally, our data provide anatomical evidence for the role of SRC-1 and SRC-2 in the E-induction of PR-A and PR-B. Our study provides a basis for further exploration into the causal roles of SRC-1 and SRC-2 in the E-induction of the two PR isoforms.
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AF</td>
<td>Activation Function</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate Nucleus</td>
</tr>
<tr>
<td>CBP</td>
<td>cAMP response element binding protein – binding protein</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA Binding Domain</td>
</tr>
<tr>
<td>E</td>
<td>Estradiol</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ERα</td>
<td>→ Estrogen Receptor α</td>
</tr>
<tr>
<td>ERβ</td>
<td>→ Estrogen Receptor β</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen Response Element</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-Releasing Hormone</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone Acetyltransferase</td>
</tr>
<tr>
<td>HPG</td>
<td>Hypothalamic-Pituitary-Gonadal</td>
</tr>
<tr>
<td>IR</td>
<td>Immunoreactive</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand Binding Domain</td>
</tr>
<tr>
<td>MPA</td>
<td>Medial Preoptic Area</td>
</tr>
<tr>
<td>NRC</td>
<td>Nuclear Receptor Coactivator</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>Progesterone</td>
</tr>
<tr>
<td>-------</td>
<td>--------------</td>
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| **PR** | Progestin Receptor  
  → **PR-A**  
  → **PR-B**  
  → Progestin Receptor A  
  → Progestin Receptor B |
| **PRBKO**  
**PRAKO** | Progestin Receptor B Knock-Out (expresses PR-A)  
Progestin Receptor A Knock-Out (expresses PR-B) |
| **SRC** | Steroid Receptor Coactivator |
| **VMN** | Ventromedial Nucleus of the Hypothalamus |
| **WT** | Wild-type |
**Introduction**

**Introduction to hormones**

Chemical messengers are molecules that are important in the mediation, synchronization, and regulation of various events in the human body, including the onset of puberty and the regulation of the menstrual cycle. In order to mediate these events, signaling molecules are released through three major pathways. Paracrine molecules diffuse between neighboring cells carrying signals from one cell to an adjacent cell (e.g. neurotransmitters). Exocrine molecules are secreted from exocrine glands into ducts that are then transported to distinct areas throughout the body. Endocrine molecules, or hormones, are secreted directly into the bloodstream where they can travel far from their gland of origin to target organs throughout the body. There are many endocrine glands in the body including the adrenal gland, the pituitary gland, and the gonads.

Endocrine glands produce a variety of classes of hormones including protein hormones and steroid hormones. Protein hormones, such as lutenizing hormone and leptin, are hydrophilic and therefore cannot pass through the lipid bilayer of cells. Thus protein hormones elicit their effects by binding to membrane-bound receptors. However steroids are lipids and therefore can easily diffuse through the lipid membranes of cells. In the classic mechanism of action, steroid hormones bind to intracellular receptors to elicit a response by altering gene expression.

Steroid hormones are classified into categories based on their gland of origin. In the case of the gonadal steroid hormones, there are three classes: (1) Estrogens (2) Progestins, and (3) Androgens. While all three classes of hormones are found in both females and males, Estrogens and Progestins are found in higher levels in females, and
Androgens are higher in males. In females, the ovaries are the major source for the synthesis and release of estrogens and progestins. The two major ovarian steroid hormones that are found endogenously in the human body are estradiol (E, $17\beta$ estradiol) and progesterone (P).

**Hormones and the brain: the physiology of synchronization**

Gonadal steroids are synthesized and released under the regulation of the hypothalamic-pituitary-gonadal (HPG) axis. The pathway of the HPG axis begins in a part of the brain called the hypothalamus. The hypothalamus is highly involved in regulatory mechanisms including the maintenance of homeostasis, feeding behavior, and reproductive behavior (Lee and Pfaff, 2008; Ladyman and Grattan, 2013). Specific neurons in the hypothalamus synthesize and release Gonadotropin-Releasing Hormone (GnRH) and are referred to as GnRH neurons. These neurons release GnRH into a network of vessels called the primary plexus in response to certain environmental stimuli such as stress, nutrition, exercise, and seasonal cues. From the primary plexus, GnRH then travels through the secondary plexus and into the anterior pituitary.

The pituitary gland, which sits on the base of the brain, consists of anterior and posterior divisions. In response to GnRH action, the anterior pituitary synthesizes and releases two hormones: (1) Follicle-stimulating hormone and (2) Lutenizing hormone. Follicle-stimulating hormone requires only GnRH to be released from the anterior pituitary, so Follicle-stimulating hormone will be released in relatively high levels in response to the GnRH stimulus. Follicle-stimulating hormone travels to the ovaries,
where it stimulates the growth of the spherical aggregation of cells around the ovum (egg) called the follicle.

The mature follicle synthesizes and releases E into the bloodstream. Together, GnRH and E stimulate the relative increase in the release of Lutenizing hormone, which acts in the ovaries to promote ovulation and the growth of the corpus luteum. The ruptured follicle becomes the corpus luteum and releases high levels of E and P. High levels of E and P travel through the bloodstream to build up the uterine lining. Additionally E and P provide negative feedback of the HPG axis at both the level of the hypothalamus and the anterior pituitary, preventing ovulation from recurring.

At this point in the cycle, a sperm cell will either fertilize the ovum, or there will be no fertilization. Upon fertilization the cells divide, forming a blastocyst that will implant onto the uterine lining or endometrium. The blastocyst then releases human chorionic gonadotropin, which maintains the corpus luteum and the endometrium. Failure of fertilization results in the degradation of the corpus luteum and a decrease in relative E and P levels, which results in the shedding of the endometrium in a process called menstruation. After menstruation, the cycle starts again.

The general HPG axis is evolutionarily conserved among mammals. Mammals experience the cyclical rise and fall of gonadal steroid hormones in response to the regulation of the HPG axis after sexual maturity. Furthermore the HPG axis of mammals regulates ovulation. However, there are key differences between the cycles of primates and other mammals. While women and other female primates build and shed an endometrium every 28 days in a process called the menstrual cycle, other mammals have a different cycle. Mice, for example, have an estrous cycle instead of a menstrual cycle.
In humans, ovulation is not completely linked with sexual behavior. In mice, the estrous cycle repeats after 3-4 days, ovulation is tightly linked with sexual behavior, and menstruation does not occur.

Despite these differences, mice are an excellent model for the study of the HPG axis and sexual behavior in human females because of the striking similarities between the neuroendocrine pathways.

**Mechanism of steroid hormone action in the brain**

Receptors for E and P are types of steroid receptors, which have a similar overall structure. Steroid receptors are modular proteins with regions of different and distinct functions (Edwards, 2005; Wierman, 2007). The N terminus of the protein contains an activation function (AF-1), which acts as a surface for transcriptional activation. The mid-region of the protein contains a DNA binding domain (DBD), where the steroid receptors binds to the promoter region of a gene, followed by a hinge region. The C-terminus of the protein contains a ligand-binding domain (LBD) where the steroid binds to the steroid receptor. Within the LBD is another activation function (AF-2), which is a transcriptional activation region that is dependant on the ligand.

Once a steroid binds to its steroid receptor there are two major mechanisms of action: the classic (genomic) pathway and the rapid non-classical pathway involving cytoplasmic kinase signaling (Mani and Blaustein, 2012). The classic pathway of steroid receptor action requires the receptor to travel to the nucleus and act as a transcription factor. The transcribed gene is then expressed through the translational machinery into a protein that can then affect other cellular processes. The non-classical pathway requires
the activated receptor to activate a kinase pathway to affect cellular processes through post-translational modifications to proteins, including phosphorylation (Toran-Allerand et al., 1999). The genomic pathway is slower than the kinase cascade, but its effects on the cell often last longer. The present study will focus on the genomic mechanism of steroid receptor action.

Through the genomic mechanism of steroid receptor action, the steroid binds to the steroid receptors and induces a conformational change. The conformational change in the steroid-bound steroid receptor results in the dissociation of various heat shock proteins from the steroid receptor, which allows two activated steroid receptors to dimerize. The steroid receptors dimer will then travel to the nucleus and bind to a steroid response element on the promoter region of certain genes. Once bound to the steroid response element, the steroid receptors will recruit various cofactors to enhance the activity of the transcriptional machinery.

E binds to its receptor ER, which has two subtypes: ERα and ERβ. While ERα and ERβ are structurally similar, they are not transcribed from the same gene (Kuiper et al., 1996). While the two subtypes of ER are co-expressed in certain target tissues, they are usually not expressed in the same areas in the brain (Shughrue et al., 1997; Greco et al., 2001; Mitra et al., 2003). ERα and ERβ have different transcriptional activity in the brain. For example, ERα is known to be a more efficient transcriptional activator than ERβ (Delaunay et al., 2000). Furthermore, as well as having different transcriptional activity, ERα and ERβ bind different estrogens (Kuiper et al., 1997).

PR are expressed as two isoforms, PR-A and PR-B, which are transcribed from the same gene (Kastner et al., 1990; Kraus et al., 1993). PR-B is the full-length protein

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while PR-A lacks the first 164 amino acids. In humans full-length PR-B is 933 amino acids while the mouse PR-B is 923 amino acids. Similarly to the subtypes of ER, PR-A and PR-B have different functions in gene transcription and reproductive behavior as discussed below (Attia et al., 2000; Mulac-Jericevic et al., 2000; Mani et al., 2006).

Hormones and the brain: behavior and relevant hypothalamic nuclei

Over the course of the estrous cycle in mice and rats, there is a relative rise in estrogens followed by a rise in progestins directly prior to ovulation. In addition to the involvement of hormones in the physiological preparations for the fertilization and implantation of an embryo, E and P are also involved in sexual behavior. The pattern of E increase followed by a peak in P elicits sexual behavior in female rats that are normally cycling. When a male rat mounts a female in estrus, the female immobilizes and arches her back into a concave C-shape (Feder, 1984). This position is called lordosis and is one indicator of female receptivity towards the sexual advances of the males.

Lordosis has been seen in normally cycling female rats around the time of ovulation, which occurs right after the peak in P. Removal of the ovaries suppresses lordosis and other displays of sexual behavior in female rodents. Studies have shown that lordosis can be recovered after the removal of the ovaries, the endogenous source of E and P, by sequential injections of E and P (Whalen, 1974; McEwen et al., 1979; Rubin and Barfield, 1983a, b). These studies confirmed that ovarian steroid hormones act in the brain to influence reproductive behavior.

E and P act in the hypothalamus to stimulate reproductive behavior in females. In order for E to induce PR, E must bind to ER and travel to the nucleus (Falkenstein et al., 2000; Schmidt et al., 2000). Another study correlates the rise of ER levels in the nucleus
with a rise of PR levels in the cytosol, where proteins are produced (Bayard et al., 1978; Parsons et al., 1981; Parsons et al., 1982). These studies provide strong evidence for the E induction of PR in the female rat hypothalamus. The nuclei of the hypothalamus that are particularly involved in reproductive behavior are the medial preoptic area (MPA), the arcuate nucleus (ARC), and the ventromedial nucleus (VMN).

The involvement of the hypothalamus in reproductive behavior has been shown through various studies. Electrical stimulation of the MPA has been shown to suppress lordosis in E-primed rats (Pfaff and Sakuma, 1979b). Lesions to the MPA have been found to increase the time of female sexual receptivity (Powers and Valenstein, 1972). Together these studies provide evidence for the inhibitory effect of the MPA on female sexual behavior and receptivity. While the MPA has been shown to inhibit female sexual behavior in rodents, the VMN has been shown to elicit such behaviors. Electrical stimulation of the VMN has been found to facilitate lordosis in E-primed rats (Pfaff and Sakuma, 1979b). Furthermore lesions to the VMN have been found to cause deficits in eliciting lordosis in E-primed rats (Pfaff and Sakuma, 1979a). Recently it has been shown that the ARC is involved in eliciting female sexual behavior in rats through the protein kinase C signaling pathway, as well as through other downstream actions of membrane estrogen receptors (Dewing et al., 2007; Dewing et al., 2008).

Estrogens and progestins work in the body to influence both physiology and behavior by binding to, and activating, their respective receptors, ER and PR. The activated receptors regulate physiology and behavior through activating kinase pathways or inducing the expression of certain genes. Increasing PR levels in the brain correlate with the increasing intensity of lordosis in female rats (Parsons et al., 1980). Conversely,
blocking PR action using a P antagonist inhibits P-induced sexual behavior in female rats (Brown and Blaustein, 1984; Etgen and Barfield, 1986). Together, these studies highlight the necessity of PR in the mediation of female sexual behavior.

Levels of PR in the brain vary significantly throughout the estrous cycle. In studies that examine sexual behavior based on ovariectomized rats with injections of E and P, the timing of administered E correlates with a rise in PR induction (Parsons et al., 1982). Further studies have observed that an increased dosage of E correlated with increased levels of PR in the female rat brain (Brown et al., 1987). These studies indicate that E induces PR production in the female rat brain.

**Estradiol induction of Progestin Receptors**

As was previously mentioned, the menstrual cycle in humans and estrous cycle in mice is defined by a peak in E secretion followed by a peak in P secretion. The timed release of E and P results in physiological changes (e.g. ovulation) and behavioral responses (e.g. sexual receptivity) in both female humans and female mice (Powers, 1970; Erskine, 1989; Penton-Voak et al., 1999; Penton-Voak and Perrett, 2000; Deschner et al., 2004; Haselton et al., 2007; Durante et al., 2008). In addition to physiological and behavioral changes in response to E release, there are also changes that occur on a subcellular level, including the expression of PR in the brain. The E induction of PR in the brain makes the brain more receptive to the peak in P levels later. Therefore, the mechanism of E-induced transcription of PR is important to reproductive physiology and behavior in females.
E induces PR gene transcription largely through ERα activity (Moffatt et al., 1998; Kudwa and Rissman, 2003). It has been shown that most PR cells in the rodent hypothalamus also express ERα providing neuroanatomical evidence for the involvement of ERα in the induction of PR (Blaustein and Turcotte, 1989; Warembourg et al., 1989). The binding of E causes a conformational change in the structure of ERα due to the dissociation of various heat shock proteins that maintain its inactive conformation (Pratt et al., 2004). This conformational change leads to the dimerization, or binding, of two activated ER proteins. The ERα homodimer then travels to the nucleus where it will bind to one of the two estrogen response elements (ERE) on PR gene (Kastner et al., 1990; Kraus et al., 1993). The expression of PR-B is controlled by an ERE upstream from the promoter region of PR gene, while the expression of PR-A is controlled by an ERE that is located within PR gene (Kastner et al., 1990; Kraus et al., 1993).

**Differences in PR-A and PR-B function**

The major difference between the two isoforms is the presence of a third activation function (AF-3) in human PR-B that allows for its interaction with transcriptional coactivators (Sartorius et al., 1994), which could potentially cause the known differences in the transcriptional activity of PR-A and PR-B (Attia et al., 2000) (Figure 1).
Figure 1. Schematic structure of mouse PR isoforms. Both PR isoforms contain an identical sequence from 165th residue onward. Within the shared sequence of both PR isoforms are two activation functions, AF-1 and AF-2, which facilitate the binding of cofactors. AF-2 also serves as a ligand-binding domain (LBD) where progesterone binds. Both PR isoforms also have an identical DNA binding domain (DBD).

The transcriptional activity of PR-A and PR-B is variable depending on the type of cell, but PR-B is generally the stronger transactivator (Vegeto et al., 1993; Tung et al., 2006). Furthermore, in certain cells in which both isoforms are expressed, human PR-A represses the transcriptional activity of human PR-B through the formation of a PR-A/PR-B heterodimer (Vegeto et al., 1993; Mohamed et al., 1994; Sartorius et al., 1994; Mulac-Jericevic et al., 2000). Due to the differential roles of PR-A and PR-B, the two isoforms occasionally have complementary roles in the regulation of gene expression. For example, in certain lines of breast cancer cells, it has been shown that an imbalance in the ratio of PR-A/PR-B induces increased transcription of genes involved in growth (Hopp et al., 2004). The balance of the different roles of PR-A and PR-B in the transcription of certain genes is
important in normally functioning mammary glands. The over-expression of either PR-A or PR-B in the mammary tissues leads to offsetting the PR-A/PR-B balance and elicits aggressive phenotypes of breast cancer (Shyamala et al., 1998; Mote et al., 2004; Khan et al., 2012).

In addition to the differential roles of PR-A and PR-B in transcribing genes that are involved in breast cancer, the PR isoforms have differing roles in the mediation of hormone-dependant sexual behavior in female mice. Mice were engineered to lack either PR-A or PR-B due to mutations in the promoter region for either isoform (Mulac-Jericevic et al., 2000; Mulac-Jericevic et al., 2003). The mice that lacked PR-B were created using a CRE/loxP-based gene targeting strategy in which the ATG start codon of PR-B was mutated in exon 1 of the mouse PR gene (Mulac-Jericevic et al., 2003). Mice that lacked PR-A were slightly harder to create because the promoter of PR-A is within the coding region of PR-B. Therefore PRAKO mice were created through the CRE/loxP-based gene targeting strategy in which there was a semi-conservative point mutation in the PR-A start codon that replaced Met^{166} with Ala in the PR gene (Mulac-Jericevic et al., 2000). This point mutation prevents the translation of PR-A, but does not interfere in the expression and functioning of PR-B (Mulac-Jericevic et al., 2000).

Using these PR-A and PR-B knockout mice (PRAKO and PRBKO respectively), Mani et al (2006) studied the effects of the different isoforms of PR on the sexual behavior of female mice. When treated with E and P, PRAKO female mice displayed significantly less lordotic activity than either PRBKO or control female mice, suggesting that PR-A may play more of a role than PR-B in eliciting female sexual behavior (Mani et al., 2006).
Role of Nuclear Receptor Coactivators in genomic effects of steroid receptors

Nuclear receptor coactivators (NRC) are proteins that are required for the efficient and enhanced transcription of genes (McKenna et al., 1999; O'Malley, 2006; Rosenfeld et al., 2006). Together, the steroid receptor dimer and NRCs form a protein complex that binds to the steroid response element of the gene. Once attached to the DNA, the protein complex initiates the transcription of the gene. NRCs enhance the transcriptional activity of steroid receptors and are thus integral to the genomic actions of steroid receptors. The first NRCs that were found to interact with steroid receptors are the members of the p160 family of proteins of which there are three members: steroid receptor coactivator-1 (SRC-1/NcoA-1), SRC-2 (GRIP1/TIF2/NcoA-2), and SRC-3 (Onate et al., 1995; Voegel et al., 1996; Suen et al., 1998).

The p160 family of nuclear receptor coactivators shares a common structure. The p160 SRCs are able to physically bind to agonist-bound receptors through many LXXLL motifs, where L is leucine and X is any amino acid. One activation domain is a binding site for other coactivators that are recruited by the members of the SRC family including CREB binding protein (CBP) (Chen et al., 1997). The second activation domain is a binding site for other proteins such as coactivator-associated arginine methyltransferase 1 (CARM1), which are the enzymes required for the unraveling of DNA from histones (Chen et al., 1999). The ability of SRC-1 and SRC-2 to increase the transcriptional activity of ER and PR specifically has been shown through in vitro studies (Kamei et al., 1996; Torchia et al., 1997). Our lab has shown that knocking down the expression of SRC-1 and CREB binding protein (CBP) resulted in decreased expression of E-induced PR in the hypothalamus of female rats (Molenda et al., 2002). Another study showed that
knocking down the expression of SRC-1 and SRC-2, but not SRC-3, resulted in decreased E-induced PR expression in the hypothalamus of female mice (Apostolakis et al., 2002). These studies show the importance of SRC-1 and SRC-2 in the expression of PR through the transcriptional activity of ERα.

SRC-1 and SRC-2 enhance the transcriptional activity of ER through a two-step model. First one SRC binds to the ERα homodimer, which then travels to the nucleus and binds to one of the two ERE on the PR gene (Kraus et al., 1993; Hong et al., 1996; Webb et al., 1998; Margeat et al., 2001). Once attached to the DNA, SRC recruits other coactivators, such as CBP, which work to stabilize protein-protein interactions in the protein complex and promotes histone acetyltransferase (HAT) activity (Lonard and O'Malley, 2005). HAT is an enzyme that transfers an acetyl group to the histones, making them more negatively charged and weakening the bonds between the histones and the negatively charged DNA. The weakened bond between the histones and DNA allows for the unraveling of DNA from the histones, which allows the general transcriptional machinery to access the gene. Certain SRCs will induce HAT activity for specific histones that are required for the efficient transcription of a certain region of DNA, making SRC-induced HAT activity specific to certain genes (Chen et al., 1997; Spencer et al., 1997).

The physical interaction between SRCs and steroid receptors is required for the function of the coactivators as transcriptional enhancers. Pull-down assays have shown that there is differential interaction of SRC-1 and SRC-2 with specific PR isoforms and ER subtypes (Molenda-Figueira et al., 2008; Yore et al., 2010). Furthermore, it has been shown that SRC and steroid receptors subtypes create specific protein complexes (Xu and
Li, 2003). The differences in HAT activity with different SRC could result in changes that alter gene expression. For example, a difference in SRC could result in the recruiting of HAT activity that exposes the promoter region for PR-A, ensuring more efficient transcription of PR-A rather than PR-B. These studies show that the difference in the type of SRC that binds to ER may be key in understanding the mechanism of PR isoform-specific expression.

**Evidence for role of coactivators in steroid hormone action in the brain**

Since coactivators need to be in the same cell as the receptors in order to act as transcriptional enhancers, using immunohistochemistry, our lab has shown the coexpression of SRC-1, CBP, and SRC-2 with ERα and E-induced PR in the brains of both female rats and mice (Teted et al., 2007; Yore et al., 2010; Tognoni et al., 2011). The co-localization of SRCs and ERα within the cell of animals that have been treated with E provides anatomical evidence for their interaction in the induction of E-induced genes. Furthermore, the co-localization of SRCs with E-induced PR within a cell of animals that have been treated with E, but not P, suggests that SRCs are involved in E-induction of PR. Since the animals were not treated with P, and therefore the PR are not activated, the co-localization of SRCs with PR cannot suggest a possible interaction between PR and SRCs, but rather the involvement of SRCs in the induction of PR. Together, these studies provide anatomical evidence for the potential interaction of SRCs with ERα in the nucleus as well as the potential involvement of SRCs in ERα-mediated induction of PR.

Several experiments have shown that SRC-1 and SRC-2, but not SRC-3 are involved in eliciting E-induced sexual behavior. Infusing the antisense strand of SRC-1
and CBP DNA directly into the VMN of female rats decreases the number of E-induced PR cells in the hypothalamus and suppresses lordosis behavior (Molenda et al., 2002). This study chose to inject antisense into the VMN because it is the region of the hypothalamus most associated with promoting female receptivity (Pfaff and Sakuma, 1979a, b). Further experimentation with antisense against SRC-1, SRC-2, and SRC-3 show decreased E-induced PR cells and suppressed lordosis behavior in female rats and mice treated with SRC-1 and SRC-2, but not SRC-3 (Apostolakis et al., 2002). These studies confirm the involvement of SRC-1 and SRC-2, but not SRC-3, in E-induced sexual behavior.

SRC-1 and CBP have also been shown to be involved in both ER and PR dependent actions in reproductive behavior. Antisense to either SRC-1 or CBP in the ventromedial nucleus of the hypothalamus (VMN) suppresses lordosis in female rats treated with high levels of E (Molenda-Figueira et al., 2006). Additionally, antisense to either SRC-1 or CBP suppressed such proceptive reproductive behaviors as hopping, darting, and ear wiggling in female rats treated with E and P when the antisense was administered to the rats around the time of P administration (Molenda-Figueira et al., 2006). The peak of P that corresponds with laboratory administered P corresponds with the PR facilitation of sexual behaviors, which are proceptive as opposed to receptive. Therefore, the data suggest that SRC-1 and CBP are involved in modulating PR-mediated sexual behavior in female rats.
Research questions

While a few studies have been able to identify functional differences between PR isoforms, they have not been able to elucidate a mechanism for the selective transcription of one PR isoform. One potential key to elucidating this mechanism may be through the role of different nuclear steroid coactivators in the ER-transactivation of the PR gene.

Our lab has provided anatomical evidence for the involvement of SRC-1 and SRC-2 in the E-induction of PR in the brain through the use of immunohistochemistry (Tetel et al., 2007; Tognoni et al., 2011). Moreover, experiments from our lab and others have shown that blocking the expression of SRC-1 and SRC-2 decreases the number of E-induced PR in the VMN of mice (Apostolakis et al., 2002; Molenda et al., 2002). However, involvement and expression of SRC-1 and SRC-2 in the expression of PR-A and PR-B has not been studied.

Our study aims to address the following questions: (1) Are PR-A and PR-B differentially expressed in the hypothalamus? (2) Are SRC-1 and SRC-2 differentially expressed with PR-A and PR-B? (3) Does the presence of SRC-1 or SRC-2 correlate with increased PR expression in the hypothalamus? To address these experimental questions, we used female transgenic mice that have been engineered to express either PR-A (PRBKO mice) or PR-B (PRAKO mice) (Mulac-Jericevic et al., 2000; Mulac-Jericevic et al., 2003). It is necessary to use PR isoform-specific knockout mice instead of separate antibodies that probe for PR-A and PR-B because the two isoforms share the same sequence. Since PR-A is just a truncated version of PR-B, any antibody that could recognize PR-A would also recognize PR-B.
Then triple label immunohistochemistry was used to determine the localization of PR, SRC-1, and SRC-2 in the hypothalamus. Co-localization of PR isoforms with SRC-1 and SRC-2 provides anatomical evidence for the involvement of SRCs in the E-induction of PR because it shows that SRC-1 and SRC-2 are present in cells that express PR in response to E treatment. Our analysis will determine whether SRC-1 and SRC-2 are differentially expressed in the PR isoform specific knockout mice brains. Differential expression of the coactivators would suggest that SRC-1 and SRC-2 are differentially involved in the transcription of the PR isoforms.

**Materials and Methods**

**Animals**

Female and male PRAKO+/- and PRBKO +/- mice, were kindly provided by Orla Conneely at Baylor College of Medicine and used for breeding purposes (Mulac-Jericevic et al., 2000; Mulac-Jericevic et al., 2003). Subsequent generations were bred and housed in the Wellesley College Animal Facility. All animals were housed in groups of 3-6 mice under 12:12hr light/dark cycle. Food and water were available *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee of Wellesley College.

**Ovariectomy and hormone treatments**

PRBKO and PRAKO mice were ovariectomized between 8 and 10 weeks of age under 1.5% isofluorane. Ovaries were removed through lateral bilateral incisions made between the pelvic bone and the bottom of the rib cage. One week after ovariectomy, mice were
injected subcutaneously with either 1µg of estradiol benzoate (EB) dissolved in 100µl of sesame oil or 100µl of just the sesame oil vehicle as a negative control. Furthermore, WT mice (+/+) for both PR-A and PR-B strains were additionally used as a control. Overall there were eight distinct groups used in this study (Table 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil</td>
<td>PRBKO</td>
</tr>
<tr>
<td>EB</td>
<td>PRBKO</td>
</tr>
<tr>
<td></td>
<td>PRAKO</td>
</tr>
<tr>
<td></td>
<td>PRBWT</td>
</tr>
<tr>
<td></td>
<td>PRAWT</td>
</tr>
</tbody>
</table>

**Perfusion and tissue collection**

Two days after EB or vehicle treatment, mice were deeply anesthetized by injecting 100µL of the anesthetic sodium pentobarbital (390mg/mL). Animals were then perfused with saline for 1 minute at a flow rate of 8 mL/minute followed by 4% paraformaldehyde (w/v) in 0.1M phosphate buffer (pH, 7.2) for 8 minutes at a flow rate of 8 mL/minute. Brains were then removed and post-fixed in the 4% paraformaldehyde solution at 4°C for 3 hours. Then the brains were transferred and stored in a 20% (w/v) glucose solution in 0.1M phosphate buffer at a pH of 7.2 for 48 hours. All brains were sectioned after 48 hours in the 20% glucose solution.

Brains were cut at 40µm thick coronal sections using a freezing microtome. Serial sections were collected from the branching of the anterior commissure (fig. 28) to the first separation of the ventral third ventricle (fig. 50) (Paxinos, 2004). The sections used for immunohistochemistry were those that were matched to figures from the mouse brain atlas for the VMN (fig. 46), ARC (fig. 46), and MPA (fig. 32) (Paxinos, 2004).
**Triple-label immunohistochemistry for PR, SRC-1, and SRC-2**

Triple-label immunohistochemistry was done to identify the specific cells that contained SRC-1, SRC-2, and E-induced PR-A and PR-B in the mouse VMN, ARC, and MPA. To quench autofluorescence due to any remaining aldehydes from fixation, the sections were first washed with 0.1M glycine in 0.05M Tris-buffered saline (TBS) for 30 minutes. Then the sections were washed in TBS. To further reduce excess paraformaldehyde, the sections were then washed with 0.5% sodium borohydride (w/v) in TBS for 20 minutes. Following additional washes in TBS, the sections were incubated in 5µg/mL of Donkey-anti-mouse Immunoglobulin G (IgG) (Jackson IR, cat #: 715-007-003) for 90 minutes in order to block against non-specific binding for the mouse monoclonal primary antibody. After further washes in TBS, the sections were incubated in TBS with 0.4% Triton-X (w/v), 10% Normal donkey serum (v/v), and 1% hydrogen peroxide (v/v) to block against non-specific binding of the secondary antibodies made in a donkey host and to quench the endogenous peroxidases.

The sections were then incubated overnight in a TBS solution in primary antibodies with 1% Normal donkey serum (v/v) for 24 hours (Table 2). The following day, the sections were first rinsed in TBS and incubated in secondary antibodies with 3% Normal donkey serum in TBS for 90 minutes (Table 2).
Table 2. Antibodies used for triple label immunohistochemistry

<table>
<thead>
<tr>
<th>Target</th>
<th>1° Antibody</th>
<th>Concentration</th>
<th>Information</th>
<th>Manufacturer</th>
<th>2° Antibody*</th>
<th>Host Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR</td>
<td>PR10A9</td>
<td>1:20</td>
<td>Mouse-anti-human PR</td>
<td>binds at AA 922-933 in human PR and 915-926 in mouse PR</td>
<td>Beckman Coulter</td>
<td>cat #: IM1408</td>
</tr>
<tr>
<td>SRC-1</td>
<td>M-20</td>
<td>1:200</td>
<td>Goat-anti-mouse SRC-1</td>
<td>binds at the C-terminal end</td>
<td>Santa Cruz Biotechnology</td>
<td>cat #: SC6098</td>
</tr>
<tr>
<td>SRC-2</td>
<td>NCOA2</td>
<td>1:800</td>
<td>Rabbit-anti-human SRC-2</td>
<td>binds at AA 1400-1464</td>
<td>Novus Biologicals</td>
<td>cat #: NB100-1756</td>
</tr>
</tbody>
</table>

*all secondary antibodies obtained from Invitrogen/Life Technologies

The sections were then rinsed thoroughly to remove any excess secondary antibody and were mounted onto slides. The slides were allowed to dry in the dark under the hood and then coverslipped with Fluorogel in Tris Buffer (Electron Microscopy Sciences, cat #: 17985-10). From the incubation in secondary antibody onward, the sections were kept in dim or dark lighting conditions at all times to prevent fluorescent bleaching. The slides were stored in the dark at 4°C until imaging.

As negative controls, primary and secondary antibody omissions were conducted. The antibody omissions tested for non-specific binding of either primary or secondary antibody. In the primary antibody omissions, there were four conditions: (1) only SRC-1 and SRC-2 antibodies (2) only PR and SRC-2 antibodies (3) only PR and SRC-1 antibodies (4) with all antibodies. In all four conditions, PR, SRC-1, and SRC-2 secondary antibodies were used. In the secondary antibody omissions there were four similar conditions, except the secondary antibodies were omitted. In all four conditions of
the secondary omissions, PR, SRC-1, and SRC-2 primary antibodies were used. In these omissions the other two sets of antibodies were used normally to ensure that the antibodies were not cross-reacting. In both the primary and secondary omissions, there was no labeling with the missing antibody, which indicates that there was no cross-reactivity in the binding between secondary antibodies and primary antibodies.

Additionally, a preadsorption assay was conducted for PR10A9 primary antibody to ensure that the antibody binds to both PR-A and PR-B. In the preadsorption assay, the PR10A9 antibody was incubated overnight with either mouse recombinant PR-A or PR-B from baculovirus transfection into insect cells. Then the antibody that was incubated with PR-A was used in immunohistochemistry for a PRBKO section and the antibody that was incubated in PR-B was used in the immunohistochemistry of a PRAKO section. Preadsorption assays were conducted previously for the SRC-1 and SRC-2 primary antibodies used in this experiment (Tognoni et al., 2011). After immunohistochemistry was done using the preadsorbed antibodies, there was no immunoreactivity. This lack of immunoreactivity indicates that the primary antibodies were already saturated with antigen, therefore our primary antibodies can bind to our proteins of interest.

**Confocal imaging of PR, SRC-1, and SRC-2 cells in the hypothalamus**

Immunoreactive cells were imaged on a Leica laser scanning confocal microscope (TCS SP5 II) with Leica software (LAS). All images were collected under 400x magnification with the PLAN-APO oil objective with a numerical aperture of 1.25. AF 488 was excited using the Argon laser set at 25% power output and 25% transmission and the emissions
were collected between 495nm and 530nm. AF 594 was excited using the HeNe 594 laser set at 40% transmission and emissions were collected between 600nm and 630nm. AF 647 was excited using the HeNe 633 laser set at 50% transmission and emissions were collected between 650nm and 700nm. The emissions were collected with three separate photomultiplier tubes (PMT). Excitation of the fluorophores was done in sequential scans with AF 594 pseudo-colored red, AF 488 pseudo-colored green, and AF 647 pseudo-colored blue. The gain and offset values for each laser were optimized and kept constant within brain regions (Table 3).

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Gain/Offset</th>
<th>Red</th>
<th>Green</th>
<th>Blue</th>
</tr>
</thead>
<tbody>
<tr>
<td>VMN</td>
<td>Gain</td>
<td>1020</td>
<td>950.8</td>
<td>1049</td>
</tr>
<tr>
<td></td>
<td>Offset %</td>
<td>-56.9</td>
<td>-25</td>
<td>-65</td>
</tr>
<tr>
<td>ARC</td>
<td>Gain</td>
<td>845</td>
<td>895</td>
<td>985</td>
</tr>
<tr>
<td></td>
<td>Offset %</td>
<td>-11</td>
<td>-25</td>
<td>-50</td>
</tr>
<tr>
<td>MPA</td>
<td>Gain</td>
<td>956</td>
<td>908</td>
<td>948</td>
</tr>
<tr>
<td></td>
<td>Offset %</td>
<td>-37</td>
<td>-32</td>
<td>-30*</td>
</tr>
</tbody>
</table>

 Sections were probed for PR-IR cells and 5 z-stacks were collected for each mouse with 1µm thickness each. All imaging was done by Kalpana Acharya.

**Analysis of confocal images**

Images were analyzed using the Nikon software NIS Elements Advanced Research version 3.22. Images were first uploaded in tiff format separately from each laser channel.
(Red, Green, and Blue). The images were then merged in RGB order and calibrated. The calibration standards were the same within each brain region but were different between brain regions. Next, the region of interests (ROI) were selected for each brain region (Table 4).

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>ROI (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VMN</td>
<td>86561</td>
</tr>
<tr>
<td>ARC</td>
<td>69044</td>
</tr>
<tr>
<td>MPA</td>
<td>171722</td>
</tr>
</tbody>
</table>

Then the images were processed with the smooth and regional maxima functions. The smooth function was chosen so as to take a neighborhood of 7x7 pixels and replace the intensity of those pixels with the average intensity of the neighborhood, thus reducing background. The smooth function also fills in the nucleolus, which would normally prevent the software from accurately counting cells. The regional maxima function compares each pixel's intensity with the intensity of surrounding pixels and eliminates any extra background, especially in channels with a low signal to noise ratio.

Next, any background staining was removed in the 3 channels (RGB) through the use of a set threshold for each channel based on the 0-255 gray scale. The threshold was set by calculating the average background intensity from each channel of images from 10 representative animals. The threshold was then set at 1-2 standard deviations away from the average intensity of the background for the 3 channels independently of each other.
and was kept constant within each region (Table 5). Any value below threshold was considered background and eliminated from the analysis. Of the three antibodies used, SRC-2, which was visualized through the blue channel had higher background for VMN and ARC, requiring higher threshold cutoffs.

Table 5. Threshold values for each channel on a 0-255 grayscale

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Red (PR)</th>
<th>Green (SRC-1)</th>
<th>Blue (SRC-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VMN</td>
<td>10</td>
<td>14</td>
<td>22</td>
</tr>
<tr>
<td>ARC</td>
<td>13</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>MPA</td>
<td>11</td>
<td>10</td>
<td>11</td>
</tr>
</tbody>
</table>

Further size (6-100µm) and circularity (0.65-1) restrictions were made in order to identify cells from background staining. Then each image was scanned using the NIS Elements program and the IR cells were identified. In cases where two cells were counted as one cell by the computer analysis, these objects were manually divided into two cells. The data from these IR cells, including number and intensity, were exported to Excel for sorting.

**Statistical analysis**

All ANOVA tests and subsequent post-hoc tests were conducted using the IBM SPSS Statistics Software Version 21. For all statistical tests, the data from the two wild-type groups, PRBWT and PRAWT, were run separately. Once the data from PRAWT and
PRBWT were determined to be similar, the two WT groups were pooled for further analysis.

**Number of E-induced PR cells:**
A two-way ANOVA with number of PR cells as the dependent factor and genotype and treatment as the fixed factors was used to analyze the number of E-induced PR immunoreactive cells in each group. Once an interaction was established, a Tukey HSD post-hoc test was used to compare groups.

**Immunostaining intensity of E-induced PR cells:**
To determine if coactivator expression influenced intensity of PR immunostaining, the PR cells were binned into 4 groups: 1) PR only cells, 2) PR cells expressing SRC-1, 3) PR cells expressing SRC-2, and 4) PR cells expressing both SRC-1 and SRC-2. A two-way ANOVA was done with PR intensity as the dependent factor and genotype and cell-type as the fixed factors. Once significance was established, a Tukey HSD post-hoc test was conducted.

**Results**

**E differentially induces PR-A and PR-B in the hypothalamus**
To investigate the E induction of PR-A and PR-B in the hypothalamus, we compared the number of PR-IR cells of EB treated mice with that of vehicle treated mice. Both PRAWT mice and PRBWT mice were used as controls and statistical analyses were performed with these as wt groups as separate controls. Because no difference was
observed between these two control groups, the two groups (PRAWT and PRBWT) were pooled. Similar to previous studies, there were E-induced PR cells in the VMN, MPA, and ARC of WT mice (MacLusky and McEwen, 1978, 1980; Parsons et al., 1980; Blaustein et al., 1988; Lauber et al., 1991; Simerly, 1993; Moffatt et al., 1998; Fenelon and Herbison, 2000; Chung et al., 2006). In the VMN there was an effect of genotype (F= 5.033, p<0.01) as well as treatment (F= 42.308, p<0.001). In the VMN, WT, PRBKO, and PRAKO mice treated with EB have greater numbers of PR-IR cells when compared with vehicle treated mice (Figure 2A). These data indicate that both PR-A and PR-B are E-induced in the VMN.

In the ARC there was an effect of genotype (F= 6.921, p<0.005) as well as treatment (F= 18.023, p<0.001) on the expression of PR-A and PR-B cells. In the ARC, both WT and PRBKO mice treated with EB have greater numbers of PR-IR cells when compared with vehicle treated mice (Figure 2B). In the PRAKO mice, there is no difference between the number of PR-IR cells in EB treated when compared with the number of PR-IR cells in the vehicle treated groups (Figure 2B). These data indicate that PR-A, but not PR-B, is E-induced in the ARC.

In the MPA there was an effect of genotype (F= 9.912, p<0.001) as well as treatment (F= 17.842, p<0.001) on the expression of PR-A and PR-B cells. In the MPA, only WT mice treated with EB have greater numbers of PR-IR cells when compared with vehicle treated mice (Figure 2C). In the MPA, although the number of PR-IR cells in PRBKO and PRAKO mice treated with EB do not differ from vehicle treated mice, there was a trend suggesting an increase in PR-IR cell number in PRBKO when treated with EB (p<0.08). These data indicate that in the MPA, E induces PR in WT mice when both
PR are expressed, but not in PRAKO or PRBKO mice when only one isoform is expressed. However, the trend towards an increase in E-induced PR-A in PRBKO mice suggests that PR-A contributes more to the E-induction of PR in the MPA of WT mice.

In addition to the main effects of genotype and treatment, there was an interaction between genotype and treatment in the VMN (F= 3.524, p<0.05), the ARC (F= 7.190, p<0.005), and the MPA (F= 6.035, p<0.005). In all three hypothalamic regions, EB treated PRAKO mice have fewer PRB-IR cells than both EB treated PRBKO (expressing PR-A) mice and WT mice, suggesting that there are fewer E-induced PR-B cells than E-induced PR-A cells in the mouse hypothalamus (Figures 2A, B, and C).
Figure 2. Differential E-induction of PR-A and PR-B in the hypothalamus. (A) In the VMN, PR-A and PR-B are E-induced. (B) In the ARC, only PR-A and PR in WT mice are E-induced. (C) In the MPA, only the PR in WT mice are E-induced. Furthermore, there was a trend of PR-A being E-induced (p<0.08). In all three hypothalamic regions, there were fewer PR-B cells than PR-A cells. * denotes within genotype differences of p<0.05 and # denotes between genotype differences of p<0.05.

EB treated WT mice have more intense PR labeling than PRAKO (expressing PR-B) mice

To investigate whether there is a difference in the relative amounts of PR-A and PR-B expressed in the female mouse hypothalamus, we examined the mean intensity of PR-IR cells in the VMN, ARC, and MPA of EB treated WT, PRBKO, and PRAKO mice. The intensity of PR-IR cells, while not indicative of the absolute amount of PR, can indicate the relative amounts of protein between genotypes. There was no difference
between PR cell intensities in either PRAWT and PRBWT for any of the hypothalamic region, so the groups were pooled into a general WT group (Data not shown). In the VMN there was an effect of genotype (F= 6.265, p<0.01) such that PR immunostaining intensity was stronger in WT mice than in PRAKO mice (Figure 3A). There was only a slight trend of increased PR immunostaining intensity in the VMN PRBKO mice when compared with PR immunostaining in PRAKO mice (p<0.087). These data support our findings above in the VMN that estradiol induces PR-A more than PR-B.

In both the ARC and the MPA there was no difference in mean intensity of PR-IR cells between genotypes, suggesting that there are the same relative amounts of PR-A and PR-B in each PR-IR cell (Figures 3B,C).
Figure 3. Intensity of PR cells of EB treated mice among all genotypes. Mean intensity of all of the PR-IR cells in the (A) VMN, (B) ARC, and (C) MPA are reported on a scale of 0-255. The dashed line represents a threshold that was set for each brain region and kept constant based on the intensity of the background (see Materials and Methods). * denotes between genotype differences of p<0.05.

Expression of SRC-1 and SRC-2 in the hypothalamus

SRC-1 and SRC-2 are widely expressed in the VMN, ARC, and MPA of PRBKO, PRAKO, and WT mice regardless of treatment (Figures 4-6). In the ARC there was an effect of genotype (F=3.479, p<0.05), but no effect of treatment on SRC-1 cell number. There were fewer SRC-1 cells in PRBWT mice than in PRAWT mice (Data not shown). Furthermore, in the ARC, there was an effect of genotype (F=4.034, p<0.05), but no effect of treatment on SRC-2 cell number. There were fewer SRC-2 cells in PRBWT
mice than in PRAWT mice (Data not shown). In the VMN and MPA there was no difference in either SRC-1 or SRC-2 cell number between genotypes. Additionally, neither SRC-1 nor SRC-2 were E-induced in the VMN, ARC, and MPA.

**Hypothalamic PR-A and PR-B cells express both SRC-1 and SRC-2**

To investigate co-localization of SRC-1 and SRC-2 with E-induced PR-A and PR-B in the brain, we used triple-label immunohistochemistry to examine the expression of PR, SRC-1, and SRC-2 in PRBKO mice and PRAKO mice. When treated with oil, there were very few PR-IR cells, but there were many cells that expressed SRC-1 and SRC-2 (Figure 4A). When treated with EB, PRBKO mice expressed PR-A immunoreactive (IR) cells only, PRAKO mice expressed PR-B IR cells only, and WT mice expressed both PR isoforms in the VMN (Figures 4-6). These findings indicate that E induces both PR isoforms in the VMN. Furthermore, in all of the genotype groups, the majority of E-induced PR co-localize with both SRC-1 and SRC-2 (Figures 4-6). Similar expression of PR, SRC-1, and SRC-2 was also found in the ARC and the MPA.
Figure 4. E-induced PR-IR cells co-localize with SRC-1 and SRC-2 in the VMN of WT mice. (A) WT mice treated with oil display an absence of PR-IR cells, but still SRC-1 and SRC-2 expression in the VMN. (B-C) WT mice treated with E display PR-IR cells that co-localize with SRC-1 and SRC-2. (A,B) Images were taken at 400x magnification. (C) Images were taken at 630x magnification. The scale bar represents 50µm.
Figure 5. E-induced PR-A IR cells co-localize with SRC-1 and SRC-2 in the VMN of PRBKO mice. The images were collected using confocal microscopy at 400x magnification. The scale bar represents 50µm.

Figure 6. E-induced PR-B IR cells also express SRC-1 and SRC-2 in the VMN of PRAKO mice. The images were collected at 400x magnification. The scale bar represents 50µm.

Majority of E-induced PR cells co-localize with both SRC-1 and SRC-2 in the hypothalamus

To investigate whether SRC-1 and SRC-2 are expressed with PR-A and PR-B, we examined the number of PR-IR cells that co-expressed SRC-1, SRC-2, both SRC-1 and SRC-2, or neither SRC-1 nor SRC-2. Since we are interested in the role of SRCs in the E-induction of PR, only EB treated mice were used for this analysis. In all three hypothalamic regions, triple-labeled cells with PR, SRC-1, and SRC-2 immunoreactivity were the most prevalent type of PR cells (Figures 7A,B,C). These data indicate that both
SRC-1 and SRC-2 are involved in the E-induction of PR-A and PR-B cells in the female mouse hypothalamus.

Figure 7. Most of the E-induced PR-IR cells in the hypothalamus also express SRC-1 and SRC-2. The proportion of E-induced PR-IR cells in the (A) VMN, (B) ARC, and (C) MPA were separated by their expression of no coactivators, SRC-1 only, SRC-2 only, or both coactivators. Only those groups that contained E-induced PR-IR cells in all three hypothalamic regions are displayed. Although PR-A cells in MPA did not differ between E-treated and oil controls, there was a strong trend towards and increase in PR-A cells. Thus, this group is also analyzed.
In WT mice, presence of SRCs increase the relative amount of E-induced PR expression in the MPA

To investigate the effect of SRCs on the E-induction of PR in the hypothalamus of female mice, we examined the mean intensity of PR-IR cells that were expressed with SRC-1, SRC-2, both SRC-1 and SRC-2, or neither SRC-1 nor SRC-2. There was no difference PR immunostaining intensity between PRAWT and PRBWT by cell type for any of the brain regions (Data not shown), so these two groups were pooled into a WT group. Since we were only interested in the genotypes that have displayed E-induced PR in the hypothalamus, we examined the mean intensities of the PR-IR cells only in those genotypes and brain regions that had E-induced PR: the 3 genotypes for the VMN and the WT and PRBKO mice for the MPA and ARC. In the VMN and the ARC, SRC-1 or SRC-2 expression did not correlate with E-induced PR-immunostaining intensity in any of the three genotypes (Figures 8A,B). However, in the MPA, the expression of both SRC-1 and SRC-2 correlated with increased intensity of PR-IR cells in WT (F= 2.541, p<0.05), but not PRBKO, mice (Figure 9). These findings suggest that both SRC-1 and SRC-2 are involved in the E-induced expression of PR, in the MPA of WT mice.
Figure 8. E-induced PR-IR cell intensity of PR cells expressed with and without SRC-1 and SRC-2 in the VMN and ARC. Mean intensity of all of the PR-IR cells in the (A) VMN and (B) ARC are reported on a scale of 0-255. The dashed line represents a threshold that was set for each brain region and kept constant based on the intensity of the background (see Materials and Methods).

Figure 9. In the MPA of WT mice, E-induces more PR-immunostaining in cells that express both SRC-1 and SRC-2. Mean intensity of all of the PR-IR cells are reported on a scale of 0-255. The dashed line represents a threshold that was set for each brain region and kept constant based on the intensity of the background (see Materials and Methods). * denotes within genotype differences of p<0.05.
Since there was a pattern of association between SRC-2 and increased PR intensity in the VMN, we further grouped the PR-IR cells by SRC-2 positive cells to see if SRC-2 positively correlated with PR intensity. When grouped by SRC-2 expression, there was a main effect of cell type on PR-IR intensity (F=6.294, p<0.005). In WT mice, PR-IR cells that were SRC-2 positive were more intensely immunostained than those PR-IR cells that expressed SRC-1 only or no coactivator at all (Figure 10A). However, when PR-IR cells were grouped by SRC-1 expression, there was no difference in the intensity of PR immunostaining (Figure 10B). These data suggest that SRC-2 contributes more than SRC-1 to the expression of E-induced PR in the VMN of WT mice. When similar analyses were done in the ARC, there were no significant difference (data not shown). When similar analyses were done in MPA both SRC-1 positive and SRC-2 positive cells had increased PR-IR cell intensity, which suggests that an increase in PR intensity was due to both SRC-1 and SRC-2 (Figures 11A,B).
Figure 10. PR-IR intensity is increased in SRC-2 positive cells in the VMN of WT mice. PR-IR cells were grouped by cells that were (A) SRC-1 positive or (B) SRC-2 positive. Mean intensity of all of the PR-IR cells are reported on a scale of 0-255. The dashed line represents a threshold that was set for each brain region and kept constant based on the intensity of the background (see Materials and Methods). * denotes within genotype differences of p<0.05.

Figure 11. PR-IR intensity is increased in both SRC-1 and SRC-2 positive cells in the MPA of WT mice. PR-IR cells were grouped by cells that were (A) SRC-1 positive or (B) SRC-2 positive. Mean intensity of all of the PR-IR cells are reported on a scale of 0-255. The dashed line represents a threshold that was set for each brain region and kept constant based on the intensity of the background (see Materials and Methods).
Discussion

Differential expression and function of PR-A and PR-B in the brain and throughout the body

The present study sought to identify the co-expression of PR-A and PR-B with two steroid receptor coactivators, SRC-1 and SRC-2, in the hypothalamus of female mice to provide anatomical evidence for the role of SRC-1 and SRC-2 in the E-induction of PR-A and PR-B. In order to investigate the expression of E-induced PR-A and PR-B with SRC-1 and SRC-2, we first treated female PRBKO and PRAKO mice with EB. Transgenic mice were used because antibodies cannot be used to differentiate PR-A from PR-B. After treating the mice with E, we probed for PR, SRC-1, and SRC-2 in three regions of the hypothalamus, the VMN, the ARC, and the MPA. These hypothalamic regions were chosen because they are known to display E-induced PR in rodents (MacLusky and McEwen, 1980; Parsons et al., 1980; Blaustein et al., 1988; Pleim et al., 1989; Warembourg et al., 1989; Turcotte and Blaustein, 1993; Brinton et al., 2008).

Through our study, we have uncovered interesting differences in the E-induction of PR-A and PR-B in the female mouse hypothalamus. Similar to previous studies, there were E-induced PR cells in the VMN, MPA, and ARC of WT mice (MacLusky and McEwen, 1978, 1980; Parsons et al., 1980; Blaustein et al., 1988; Lauber et al., 1991; Simerly, 1993; Moffatt et al., 1998; Fenelon and Herbison, 2000; Chung et al., 2006). In the VMN, both PR-A and PR-B are E-induced, in the ARC only PR-A is E-induced, and in the MPA there is only a trend of PR-A induction by E. In all three hypothalamic regions there are more PR-A cells than PR-B cells. Furthermore, PR cells in the VMN of WT mice showed more intense immunostaining than PR-B cells in the VMN of PRAKO mice, which suggests that there was more expression of PR in cells that contain both
isoforms when compared with cells that just contained PR-B. Additionally, there was a trend of increased immunostaining of PR-A cells in the VMN of PRBKO mice when compared with PR-B cells in the VMN of PRAKO mice, which suggests that there was more expression of PR-A than PR-B within individual cells in the VMN. Together, these results suggest that PR-A is the more dominant PR isoforms, compared with PR-B, in the female mouse hypothalamus. Our results also suggest that PR-A is E-induced in the ARC and MPA while PR-B is not.

These results are novel, as this is the first study to examine in situ expression of the PR isoforms using isoform specific KO mice. Furthermore, few studies have looked at the E-induction of PR-A and PR-B separately in the brain (Guerra-Araiza et al., 2003; Mani et al., 2006). One study using PR isoform specific KO mice analyzed the overall expression of E-induced PR-A and PR-B in the hypothalamus (Mani et al., 2006). The primary focus of this study was to analyze the difference in PR-A and PR-B in mediating P-dependent sexual receptivity in female mice, though PR isoform expression in the hypothalamus was also analyzed. Using western blot analysis, Mani et al showed that E-induced PR-A and PR-B were expressed in the same relative amounts in the hypothalamus. Furthermore, their western blot analysis showed that both PR-A and PR-B were E-induced in the hypothalamus. Our results do not agree with the findings of the Mani study with regard to PR isoforms expression.

A second study used the difference in molecular weight of PR-A and PR-B to analyze the relative expression of the two isoforms across the estrous cycle of female rats through the use of Western Blot analysis (Guerra-Araiza et al., 2003). In their 2003 study, Guerra-Araiza (2003) found that both PR-A and PR-B were E-induced in the
hypothalamus and the pre-optic area while only PR-A was E-induced in the hippocampus (Guerra-Araiza et al., 2003). Compared to results using western blots, in our study we provide more sensitive information on protein expression since immunohistochemistry can localize proteins on a cellular level. Furthermore, the Guerra-Araiza study was done with rats while our study was conducted with mice so there may be some species differences in PR isoform expression. Importantly, the data from our study largely agrees with the findings of the Guerra-Araiza study. However, we observed E-induction of PR-B in the VMN only and not in the preoptic area.

While there are very few studies that have looked at the differential expression of the PR isoforms in the brain, other studies have looked at the differential expression of PR isoforms in other tissues. In the mouse and human uterus, it has been shown that PR-A is expressed more than PR-B (Mangal et al., 1997; Mote et al., 1999). Furthermore it has been documented that PR-A, but not PR-B, is E-induced in the mouse uterus (Mote et al., 1999). The Mote et al. (1999) study used two antibodies that have been previously characterized to probe for PR-A and PR-B separately for use in immunohistochemistry (Clarke et al., 1987). The tissue was first incubated with the antibody for PR-B, which was selective because of the additional region on the N-terminus that is unique to PR-B. Then the tissue was incubated with an antibody that recognizes both PR isoforms, but binds to a part of the protein that is close to that of the PR-B specific antibody so the antibody can only bind to PR that have not already been labeled with the first antibody. Therefore the first antibody specifically bound to PR-B and the second antibody bound with whatever PR is remaining, PR-A (Clarke et al., 1987). These results that suggest that
PR-A is the more dominant isoform of PR in the uterus are similar to the results from our study regarding PR-A expression in the hypothalamus.

In addition to differences in expression, PR isoforms have also been shown to have functional differences in the mouse brain. Using the same PR isoform specific KO mice as in the current study, Mani et al characterized the female sexual behavior of mice expressing either PR-A or PR-B (Mani et al., 2006). After treatment with E and P, mice that only expressed PR-B exhibited decreased female sexual behavior when compared to mice that expressed either PR-A or both PR isoforms. These results suggest that PR-A is more involved in mediating female sexual behavior than PR-B, which may be due to the dominance of PR-A expression in the hypothalamus as shown in our study.

In our study, the VMN is the only region in the hypothalamus where both PR-A and PR-B are E-induced. Additionally, the VMN is the only area where PR-A has more intense immunostaining than PR-B, suggesting higher levels of PR-A expression than PR-B. One possible reason to explain this discrepancy between the VMN and the other hypothalamic regions is that the VMN is necessary and sufficient for the induction of female sexual behavior in rodents (Malsbury et al., 1978; Pfaff and Sakuma, 1979a, b; Rubin and Barfield, 1983a; Meisel et al., 1987; Pleim et al., 1989; Musatov et al., 2006). Prior to ovulation, E levels in the VMN rise and induce the expression of PR, which increases the sensitivity of the VMN to P. Following the rise in E levels, there is a subsequent rise in P levels, which induces female sexual behavior in rodents corresponding to the point in ovulation where the female is most likely to be impregnated. Since the E induction of PR increases the sensitivity of the VMN to P,
inducing both PR isoforms in the VMN, instead of just one isoform, would be evolutionarily beneficial in inducing sexual behavior in normally cycling female.

However, in the MPA, the difference between PR-A cells in the EB treated mice and oil treated mice is just a trend. A possible reason for why PR-A is not E-induced in the MPA is that there were fewer PRBKO mice in our study than PRAKO and WT mice, which led to decreased power in our statistical tests.

**Coexpression of PR-A and PR-B with SRC-1 and SRC-2 in the hypothalamus**

A previous study from our lab has already shown that E-induced PR in WT mice co-localize with both SRC-1 and SRC-2 in the hypothalamus (Tognoni et al., 2011). The current study also revealed interesting findings about the expression of two steroid receptor coactivators, SRC-1 and SRC-2, with PR-A and PR-B using transgenic mice. Through the analysis of the proportion of PR cells that co-localized with SRCs, we have shown that most of the PR-A and PR-B cells also express both SRC-1 and SRC-2. Furthermore, there was more E-induced PR in cells that also expressed SRC-2 in the VMN of WT mice. Additionally, in the MPA, there was more E-induced PR in each PR cell of WT mice when the cell also expressed SRC-1 and SRC-2. These data provide indirect evidence for the involvement of steroid receptor coactivators in the E-induction of PR. These data suggest that SRC-2 may be more involved in mediating the E-induction of PR in the VMN, which is the region that is most correlated with female sexual behavior, while both SRC-1 and SRC-2 may be more involved in the E-induction of PR in the MPA.
Our findings also agree with previous data from our lab, which have shown both the co-localization of steroid receptor coactivators with PR as well as the interaction between ERα and steroid receptor coactivators (Tetel et al., 2007; Yore et al., 2010; Tognoni et al., 2011). Co-localization studies are important to show that certain activities found in vitro can occur in vivo because the proteins involved are located in the same cells. Through the use of immunohistochemistry, it has been shown that most PR cells in the rodent hypothalamus also express ERα providing neuroanatomical evidence for the involvement of ERα in the induction of PR (Blaustein and Turcotte, 1989; Warembourg et al., 1989).

Studies from our lab have gone further to show that ERα cells in the hypothalamus also express SRC-1 and SRC-2 (Tetel et al., 2007; Yore et al., 2010; Tognoni et al., 2011). Furthermore, ERα, when bound to E, has been shown to physically interact with both SRC-1 and SRC-2 in vitro through the use of pull-down assays (Molenda-Figueira et al., 2008; Yore et al., 2010). These results suggest that the SRCs are involved in the E induced transcriptional activity of ERα. Another study from our lab has shown that blocking the expression of SRC-1 and cAMP response element binding protein - binding protein (CBP) also blocks the expression of E-induced PR in the VMN of rats (Molenda et al., 2002). A similar study showed that knocking down the expression of SRC-1 and SRC-2 decreased female sexual behavior by preventing the E-induction of PR (Apostolakis et al., 2002). The evidence from these studies combined with our data suggest a role of both SRC-1 and SRC-2 in the E-induction of PR-A and PR-B.
Further experimentation and implications

Further experimentation is needed to show whether SRC-1 and SRC-2 are differentially involved in the E-induced expression of PR-A and PR-B in the female mouse hypothalamus. To investigate whether SRC-1 or SRC-2 contributes more to the E-induced expression of PR-A and PR-B, we will stereotaxically inject antisense oligonucleotides for either SRC-1 or SRC-2 into the hypothalamus of PRAKO and PRBKO mice. Previous studies that have knocked down the expression of SRC-1 and SRC-2 suggest that the two coactivators play a role in the expression of E-induced PR, but these studies have only been done in WT mice (Apostolakis et al., 2002). Our future study will provide evidence for the specific causal evidence for the roles of SRC-1 and SRC-2 in the expression of PR-A and PR-B.

The antisense oligonucleotide will form a complex with its complementary mRNA and prevent its expression. If the steroid receptor coactivator mediates the E-induction of PR-A, then we should observe a decreased number of E-induced PR-A in antisense treated PRBKO mice when compared to control treated PRBKO mice. The proposed future study can directly address the hypothesis that SRC-1 and SRC-2 differentially contribute to the E-induction of PR-A and PR-B.

The findings from the present study and the proposed future study can further elucidate the mechanism of E-induced PR isoforms expression in the hypothalamus. The present study provides evidence for the dominance of PR-A expression in the hypothalamus. Furthermore, the study provides neuroanatomical evidence for the role of steroid receptor coactivators in the increased expression of E-induced PR in the
hypothalamus. Overall, our findings provide a basis for future studies to investigate the role of SRCs in the expression of PR isoforms.

Previous studies have already provided a rationale for the involvement of SRCs in the differential transcription of PR-A and PR-B. One possible mechanism of the role of SRCs in differential transcription is through HAT activity. Once bound to steroid receptor dimers in the nucleus, SRCs are involved in recruiting other cofactors such as CBP and p300/CBP-associated factor to form a coactivator complex. Some of the recruited cofactors such as p300/CBP-associated factor possess HAT activity (Lonard and O'Malley, 2005), which is required for the unraveling of DNA from a histone for efficient transcription of a gene (McKenna et al., 1999; O'Malley, 2006; Rosenfeld et al., 2006). In addition to the cofactors, some SRCs including SRC-1 and SRC-3 are known to have intrinsic HAT activity (Chen et al., 1997; Spencer et al., 1997). SRC-1 is known to selectively acetylate histones H3 and H4 (Spencer et al., 1997). Furthermore different steroid receptor dimers can recruit different coactivator complexes based on the type of SRC that initially binds to the dimer (Li et al., 2003). In one study, PR was found to selectively recruit SRC-1 and CBP resulting in the acetylation of histone H4, while glucocorticoid receptor selectively recruited SRC-2 and p300/CBP-associated factor resulting in the acetylation of histone H3 (Li et al., 2003). Since SRCs are known to selectively acetylate certain histones when recruited as a part of the coactivator complex (Li et al., 2003), the involvement of SRCs is a determining factor in the efficiency of transcription. Furthermore, the acetylation of specific histones could lead to SRCs controlling the location of transcription initiation. The selective acetylation of different
histones based on the recruitment of different SRCs by ERα could potentially explain how PR-A and PR-B are differentially expressed.

Further study of PR isoform-specific expression is important in elucidating the molecular mechanism of sexual behavior in women. Since the mouse estrous cycle is functionally similar to the human menstrual cycle, and both induce sexual behavior near ovulation, our findings from mouse studies may be applicable to understanding human sexual behavior (Powers, 1970; Erskine, 1989; Penton-Voak et al., 1999; Penton-Voak and Perrett, 2000; Deschner et al., 2004; Haselton et al., 2007; Durante et al., 2008).

Furthermore, elucidating the mechanism of PR isoform expression in the brain may lead to insights regarding PR expression throughout the body. For example, it is known that abnormal ratio of PR-A/PR-B in breast cancer cells decreases the prognosis for individuals with breast cancer (Hopp et al., 2004). If we can discover how PR-A and PR-B are specifically transcribed, that may lead to new targets for therapeutics for progesterone-sensitive breast cancer.

Currently selective estrogen receptor modulators, such as tamoxifen, are commonly used in the treatment of E-sensitive tumors due to their antagonistic effects on ERα. However, tamoxifen is not an antagonist for ERα in all tissues, such as the endometrium where it acts as an agonist (MacNab et al., 1984; Satyaswaroop et al., 1984; Gottardis et al., 1988). Tamoxifen works by binding to the LBD of ERα and causing a conformational change that does not allow for transcription to occur (Shiau et al., 1998). In tissues where it acts like an agonist, tamoxifen induces a conformational change in ERα that still allows it to dimerize and bind to specific ERE on DNA. Recent studies have shown that a non-receptor tyrosine kinase phosphorylates ERα bound with
tamoxifen and stabilizes the binding of SRC-1 and CBP to promote the transcription of ERα-controlled genes (Shah and Rowan, 2005).

One potential therapeutic target that may arise from research into the differential roles of SRCs in the transcription of PR-A and PR-B is the potential development of pharmacological molecules that induce a conformational change in ERα that promote the binding of one SRC selectively. Since studies have shown that different SRCs are involved in acetylating specific histones (Chen et al., 1997; Spencer et al., 1997; Li et al., 2003), recruiting certain SRCs could lead to the preferential transcription of one PR isoform over the other. Therefore such a therapeutic that leads to the preferred binding of one SRC could lead to the transcription of one PR isoform that can correct the imbalance in the PR-A/PR-B ratio in breast cancer cells and lead to an improved prognosis in patients. The ramifications of this study and future investigations have the potential to enhance our understanding of the ERα-mediated transcription of PR-A and PR-B in the brain and throughout the body.
Literature Cited


