

Nuclear Receptor Coactivators Are Coexpressed with Steroid Receptors and Regulated by Estradiol in Mouse Brain

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Key Words

Estrogen receptor · Hypothalamus · Progesterin receptor · Sexual behavior · Steroid receptor coactivator · Steroid receptor coactivator-1

Abstract

Background/Aims: The steroid hormones, including estradiol (E) and progesterone, act in the brain to regulate female reproductive behavior and physiology. These hormones mediate many of their biological effects by binding to their respective intracellular receptors. The receptors for estrogens (ER) and progestins (PR) interact with nuclear receptor coactivators to initiate transcription of steroid-responsive genes. Work from our laboratory and others reveals that nuclear receptor coactivators, including steroid receptor coactivator-1 (SRC-1) and SRC-2, function in brain to modulate ER-mediated induction of the PR gene and hormone-dependent behaviors. In order for steroid receptors and coactivators to function together, both must be expressed in the same cells. **Methods:** Triple-label immunofluorescence was used to determine if E-induced PR cells also express SRC-1 or SRC-2 in reproductively relevant brain regions of the female mouse. **Results:** The majority of E-induced PR cells in the medial preoptic area (61%), ventromedial nucleus of the hypothalamus (63%) and arcuate nucleus (76%) coexpressed both SRC-1 and SRC-2. A smaller proportion of PR cells expressed either SRC-1 or SRC-2, while a few PR cells expressed neither coactivator. In addition, compared to control ani-

mals, 17 β -estradiol benzoate (EB) treatment increased SRC-1 levels in the arcuate nucleus, but not the medial preoptic area or the ventromedial nucleus of the hypothalamus. EB did not alter SRC-2 expression in any of the three brain regions analyzed. **Conclusions:** Taken together, the present findings identify a population of cells in which steroid receptors and nuclear receptor coactivators may interact to modulate steroid sensitivity in brain and regulate hormone-dependent behaviors in female mice. Given that cell culture studies reveal that SRC-1 and SRC-2 can mediate distinct steroid-signaling pathways, the present findings suggest that steroids can produce a variety of complex responses in these specialized brain cells.

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Introduction

The ovarian hormones estradiol (E) and progesterone regulate growth, proliferation and differentiation in a variety of tissues. In addition, these hormones play an important role in hormone-dependent cancers of the reproductive systems, including breast cancer [1]. E and progesterone act in specific brain regions to influence a variety of functions, including cognition and female reproductive behavior and physiology [2, 3].

Estrogens and progestins bind to their respective steroid receptors, ER and PR, which are members of a superfamily of ligand-dependent transcription factors. In-

tracellular ER exist as two subtypes, α and β , which are transcribed from different genes [4–6]. In primates and rodents, PR are expressed in two forms; the full-length PR-B and the N-terminal truncated PR-A, which are encoded by the same gene but are regulated by different promoters [7]. In the traditional genomic mechanism of action, these steroid receptors bind hormones and undergo a conformational change that causes the dissociation of heat shock proteins and other immunophilins [8]. Activated receptors dimerize and bind preferentially to specific hormone response elements in the promoter regions of target genes to increase or decrease gene transcription [9, 10]. In addition, ER and PR have been found to function in the absence of ligand and at the membrane to rapidly activate cytoplasmic signaling pathways [11–15].

A classic example of a steroid-induced gene is the induction of the PR gene by E in a variety of tissues, including brain. This ER-mediated induction of PR is thought to occur via an estrogen response element in the promoter region of the PR gene [16–18]. While PR are present in low levels in the brains of ovariectomized rodents, E priming dramatically increases the expression of PR in the medial preoptic area (MPA), arcuate nucleus (ARC) and ventromedial nucleus of the hypothalamus (VMN) [19–28]. Based on studies in ER knockout mice, E induction of PR in brain appears to be predominantly, while not solely [29], dependent on ER α [30–32]. In support of this ER α -mediated event, virtually all E-induced PR cells in the hypothalamus also express ER α [26, 33]. These E-induced PR in the hypothalamus are important for progesterone-facilitated reproductive behavior [34]. In addition, studies using PR-A- and PR-B-specific knockouts reveal that while both receptors are important, PR-A appears to have a larger role in the full display of progesterone-facilitated lordosis [35].

Nuclear receptor coregulators consist of coactivators and corepressors that are required for efficient transcriptional regulation by nuclear receptors [36–39]. There is mounting evidence that coregulators are involved in human disease, including metabolic disorders and cancers [40]. Nuclear receptor coactivators dramatically enhance the transcriptional activity of nuclear receptors, including PR and ER, through a variety of mechanisms, including acetylation, methylation, phosphorylation and chromatin remodeling [36, 37]. In vitro, these coactivators are often rate limiting for steroid receptor activation and act as bridging proteins between the receptor and the basal transcriptional machinery [36, 37].

The p160 steroid receptor coactivator (SRC) family includes SRC-1/NcoA-1 [41], SRC-2/TIF2/GRIPI/NcoA2 [42, 43], and SRC-3/p/CIP/ACTR/AIB1/TRAM-1/RAC3 [44, 45]. Recent work reveals that two members of this p160 family of coactivators, SRC-1 and SRC-2, are important for hormone action in brain and behavior [46, 47]. SRC-1 [48–57] and SRC-2 [58–60] are expressed at high levels in the cortex, hypothalamus and hippocampus of rodents. Our laboratory and others have found that SRC-1 and SRC-2 are important for hormone-dependent sexual differentiation of the brain [53], gene expression in brain [54, 61–63] and sexual behavior [54, 61–64]. Finally, SRC-1 and SRC-2 from rodent brain physically interact with ER and PR in a receptor subtype- and brain region-specific manner [58, 65].

In order for coactivators to function with steroid receptors in hormone action in brain, both the coactivators and receptors must be expressed in the same cells. Our previous work in female rats reveals that the majority of E-induced PR cells in the hypothalamus also express SRC-1 [48]. In support, SRC-1 and SRC-2 expression is significantly related to PR expression in hormone-responsive meningiomas [66]. In contrast, SRC-1 was not detected in mouse mammary epithelial cells expressing E-induced PR [67], suggesting that SRC-1 functions in a cell-type- and tissue-specific manner. However, it is not known if SRC-1 or SRC-2 are expressed in PR-containing cells in mouse brain. Therefore, we used a triple-label immunofluorescent technique to ask if E-induced PR cells expressed SRC-1, SRC-2 or both coactivators in reproductively relevant brain regions of female mice.

Materials and Methods

Animals

Female C57 mice, 5–6 weeks old, were obtained from Taconic (Germantown, N.Y., USA) and group-housed for 1 week under a 12:12-hour light/dark cycle with food and water freely available. One week after arrival, the animals were ovariectomized under 1.5% isoflurane. One week following ovariectomy, the mice were injected subcutaneously with either 17 β -estradiol benzoate (EB, 1 μ g in sesame oil) or vehicle 48 h prior to sacrifice. Ovariectomized mice treated with EB (n = 8) or vehicle (n = 7) were anesthetized with Fatal Plus (sodium pentobarbital 0.1 ml, 390 mg/ml) and perfused with 4% paraformaldehyde. Five thousand units of sodium heparin dissolved in saline were injected into the left ventricle. Saline (0.15 M, 8 ml) preceded the flow of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH = 7.2) at a flow rate of 8 ml/min for 8 min. Brains were removed from the cranium, blocked and stored in 0.1 M sodium phosphate buffer (pH = 7.2) containing 20% sucrose at 4°C for 48 h. Coronal sections were cut on a freezing rotary microtome at 40 μ m from the MPA through

the hypothalamus following the mouse brain atlas [68]. The sections were stored in cryoprotectant at -20°C until processing. All animal procedures were approved by the Institutional Animal Care and Use Committees of Wellesley College.

Immunohistochemistry

A triple-label immunohistochemistry technique was used to identify cells expressing PR, SRC-1 and SRC-2 in the VMN, ARC and MPA. The brain sections were incubated in 0.05 M Tris-buffered saline (TBS) and incubated in donkey anti-mouse IgG to occupy the endogenous mouse antibodies. The sections were washed again in TBS and incubated in 20% donkey serum to reduce non-specific binding. To detect PR, SRC-1 and SRC-2, the sections were incubated for 24 h at 4°C in a cocktail containing a PR mouse monoclonal antibody directed against amino acids 922–933 of the C-terminus of human PR (1:6,000, MAB 462, Millipore), an SRC-1 goat polyclonal antibody directed against the C-terminus (aa 1355–1405) mouse SRC-1 (1:250, M-20, sc-6098, Santa Cruz Biotechnology), and an SRC-2 rabbit polyclonal antibody directed against the C-terminus (aa 1400–1464) of human SRC-2 (1:1,000, NB100-1756, Novus Biologicals). The specificities of the PR and SRC-1 antibodies have been established previously in rodent brain [48, 69, 70]. Analysis of homogenates of mouse hypothalamus by Western blot using NB100-1756 revealed a distinct immunoreactive band for SRC-2 (see online supplementary figure 1, www.karger.com/doi/10.1159/000323780) at the expected molecular mass of 160 kDa [41, 42]. The sections were washed with TBS and incubated in a cocktail of fluorescently labeled secondary antisera containing donkey anti-mouse serum (1:300, Alexa 594, Invitrogen) for detection of PR, donkey anti-goat (1:100, Alexa 488) for detection of SRC-1, and donkey anti-rabbit (1:100, Alexa 647) for detection of SRC-2. The sections were then washed with TBS and mounted on gel-coated glass slides, coverslipped with Fluoro-Gel (EMS) and stored at 4°C .

Controls for this triple-label technique included the omission of the primary or secondary antibodies. In addition, primary antibodies were preadsorbed with 20-fold molar excess of SRC-1 peptide of the C-terminus (SRC-1 M-20 P, Santa Cruz Biotechnology), a fragment of GST-tagged recombinant human SRC-2 protein consisting of aa 1365–1465 (H00010499-Q01, Novus Biologicals) or full-length human PR-B protein. Recombinant human PR-B proteins were expressed in *Spodoptera frugiperda* (Sf9) insect cells from the Baculovirus/Monoclonal Antibody Facility at the Baylor College of Medicine, as described previously [71, 72].

Imaging by Confocal Microscopy and Analysis

The MPA (fig. 33 of [68]), VMN (fig. 46 of [68]) and ARC (fig. 46 of [68]), which are rich in E-induced PR, were analyzed with the experimenter blind to treatment groups. Images of immunofluorescence from one section per brain region were captured at $200\times$ with a Leica TCS SP laser scanning confocal microscope (equipped with an argon laser 488, diode laser 561 and helium-neon laser 633) using imaging software (LCS 1347a, Leica). For each brain region, a two-dimensional $1\text{-}\mu\text{m}$ optical section (512×512) was captured and analyzed. One side of a representative section of each animal was analyzed using a uniform region of interest for the MPA (total area of the region of interest = $44,981 \mu\text{m}^2$), VMN (total area = $40,373 \mu\text{m}^2$) and ARC (total area = $59,809 \mu\text{m}^2$). For each brain region, the region of interest was placed over the highest concentration of PR-immunoreactive

(IR) cells in EB-treated animals and the corresponding area for vehicle animals. Laser output (mV) was measured using a Field-Master (Coherent) and kept constant between animals and imaging sessions. Triple-labeled images (8-bit) were analyzed using NIS Elements (Nikon). The threshold for detection of specific immunoreactivity was determined as a function of background. For each brain region, the threshold was established as the mean maximum pixel intensity (ranging from 0 to 256) of 10 random samples of background for 3 randomly selected animals in each group. Within each optical section, cells were considered immunopositive if above the threshold value and the total area was greater than $8.0 \mu\text{m}^2$. To insure unbiased data collection for each optical section of a brain region, all objects that met the established criteria were counted in a uniform region of interest. In each brain region, the number of immunoreactive cells and the average optical density were collected for PR immunoreactivity, SRC-1 immunoreactivity and SRC-2 immunoreactivity.

Statistical Analysis

To determine if EB influenced the expression of SRC-1 or SRC-2, images of matched sections from EB-treated and control animals were analyzed for total cell counts and relative optical density for coactivator immunoreactivity using NIS Elements (Nikon). Because the data were not normally distributed, differences in coactivator immunoreactivity between the EB and control groups were compared using a Kruskal-Wallis one-way analysis of variance by rank test (Statistica). Differences were considered statistically significant at a probability <0.05 .

Results

Coexpression of E-Induced PR, SRC-1 and SRC-2

Consistent with previous studies in mice and rats, many E-induced PR-IR cells were detected in EB-treated mice, while little to no PR were observed in control animals [20, 21, 23, 25, 30, 31, 34, 73–78] in the VMN ($EB = 35.0 \pm 2.0$ cells vs. oil = 0.3 ± 0.2 ; $p < 0.001$), ARC (109.3 ± 23.7 vs. 1.6 ± 1.4 ; $p < 0.01$) and MPA (139.0 ± 25.9 vs. 31.1 ± 17.1 ; $p < 0.01$) (fig. 1a, b, 2a). Similar differences in the average optical density of PR immunoreactivity were found between EB and control animals (data not shown).

SRC1-IR cells were observed throughout the hippocampus, amygdala (data not shown) and hypothalamus, including the VMN, ARC and MPA (fig. 1b, f, 2b), which is consistent with other limited studies of SRC-1 mRNA in mice [59, 79]. In addition, the present findings of SRC-1 expression in mouse brain are consistent with previous studies in rats [48–54, 70, 80] and birds [57, 81]. SRC2-IR cells were detected throughout the mouse hippocampus, amygdala (data not shown) and hypothalamus, including the VMN, ARC and MPA (fig. 1c, g, 2c). While the pattern of SRC-2 expression in brain has been studied much less, these results are consistent with other findings in mice and rats [59–61].

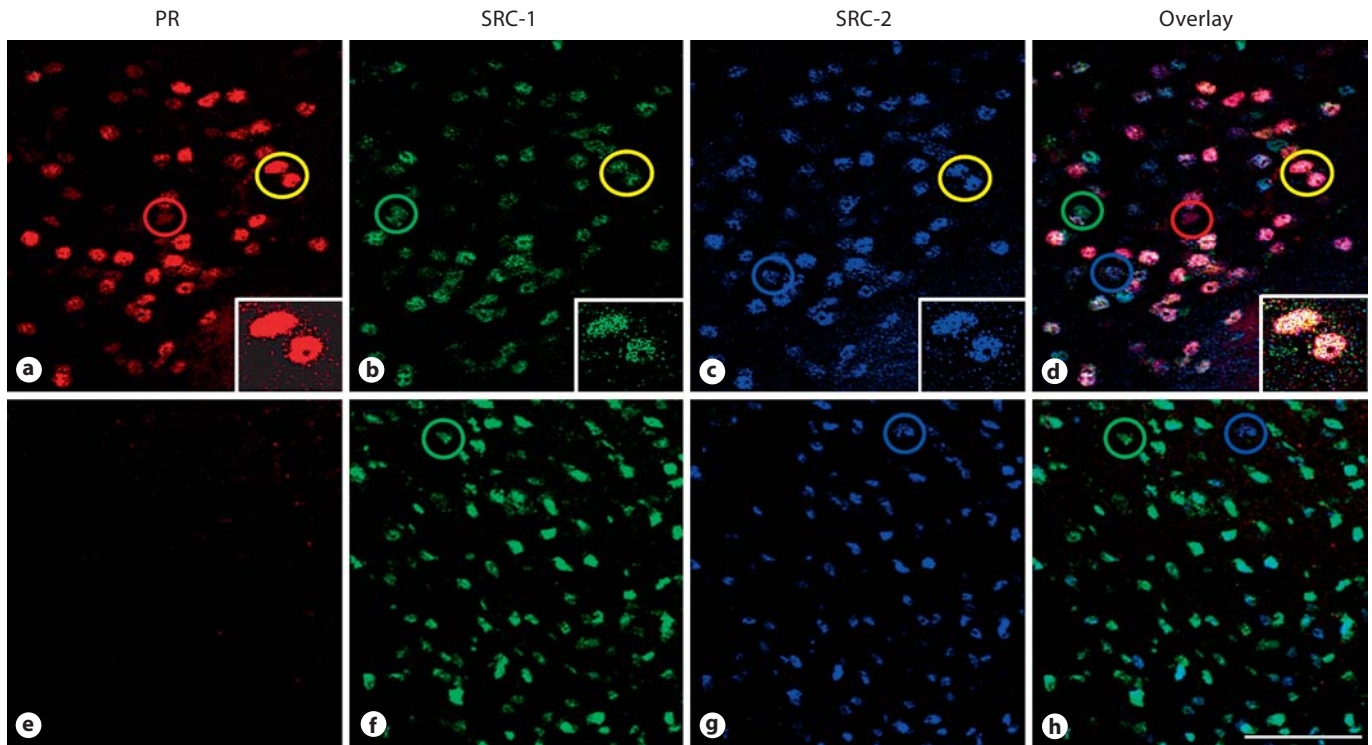


Fig. 1. E-induced PR cells coexpress SRC-1 and SRC-2 in the VMN of female mice. The VMN of animals treated with EB (**a–d**) or vehicle (**e–h**) were immunostained for PR (**a, e**), SRC-1 (**b, f**), or SRC-2 (**c, g**). **d, h** Overlaid image shows cells expressing PR, SRC-1 and SRC-2 (yellow circles), shown in inset at higher magnification. Red circles indicate a cell expressing PR only, green circles indicate a cell expressing SRC-1 only and blue circles indicate a cell expressing SRC-2 only. Bar = 50 μm .

Table 1. Cells immunostained for PR, SRC-1 and SRC-2 in the mouse VMN, ARC and MPA

Brain region	Total			% PR cells expressing				% SRC-1 cells expressing PR	% SRC-2 cells expressing PR
	PR cells	SRC-1 cells	SRC-2 cells	neither SRC-1 nor SRC-2	SRC-1 only	SRC-2 only	SRC-1 and SRC-2		
VMN	35 \pm 2.0	39 \pm 6	33 \pm 7	12	18	7	63	78	82
ARC	109 \pm 22	232 \pm 15	195 \pm 25	3	18	2	76	44	45
MPA	139 \pm 24	250 \pm 29	235 \pm 34	7	19	13	61	44	44

Total numbers of cells per region of interest for each brain region are shown as the mean \pm SEM.

We found that a large majority of cells containing E-induced PR in the VMN coexpressed both SRC-1 and SRC-2 (table 1; fig. 1d). In addition, most of the E-induced PR cells in the MPA and ARC coexpressed both SRC-1 and SRC-2 (table 1; fig. 2d). A smaller proportion of E-induced PR cells in these three brain regions contained only SRC-1. Interestingly, in all three brain regions, fewer

PR cells expressed SRC-2 only than SRC-1 only (table 1). A relatively small population of E-induced PR-IR cells expressed neither of the coactivators. In the VMN, the majority of coactivator-expressing cells also expressed PR (table 1; fig. 1d). However, in contrast to the VMN, the majority of SRC1-IR and SRC2-IR cells in the ARC and MPA did not express E-induced PR (table 1).

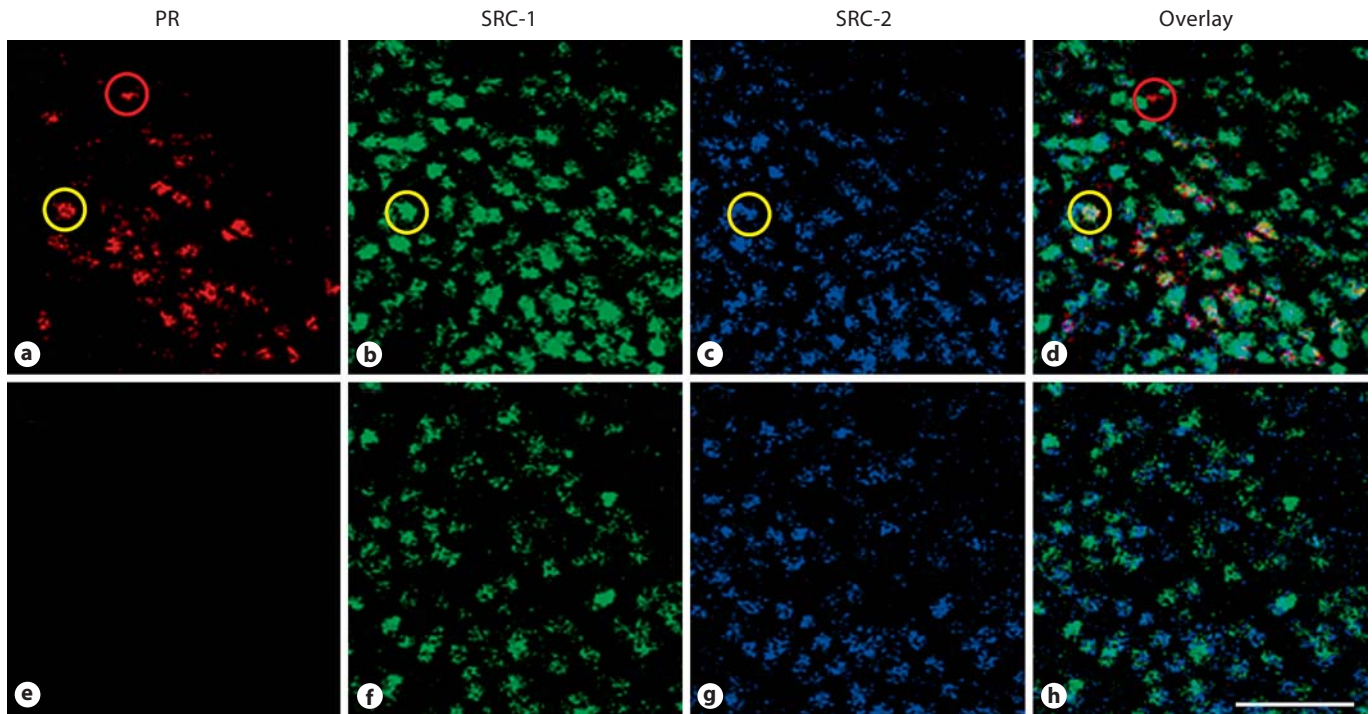
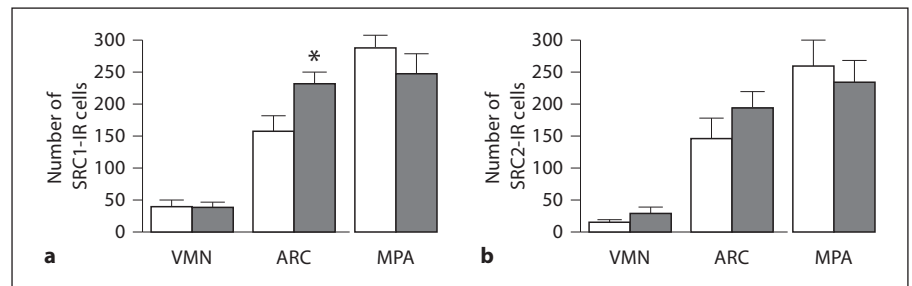


Fig. 2. Expression of PR, SRC-1 and SRC-2 in the ARC of female mice treated with EB or vehicle. The ARC of animals treated with EB (a–d) or vehicle (e–h) were immunostained for PR (a, e), SRC-

1 (b, f), or SRC-2 (c, g). Red circles indicate a cell expressing PR only and yellow circles indicate a cell expressing PR, SRC-1 and SRC-2. Bar = 50 μ m.

Fig. 3. EB regulates SRC-1 expression in the ARC. Average number (\pm SEM) of cells immunoreactive for SRC-1 (a) or SRC-2 (b) per region of interest for the VMN, ARC and MPA from oil- (open bars) and EB- (grey bars) treated mice. * $p < 0.02$.



Regulation of Nuclear Receptor Coactivator Expression by Estradiol

To determine if EB alters the expression of SRC-1 or SRC-2 expression in the VMN, MPA or ARC, sections from EB- and vehicle-treated animals were compared. EB increased the number of SRC1-IR cells in the ARC, but not in the MPA or VMN (fig. 2b, f, 3a). No differences were detected in the number of SRC2-IR cells between EB and control animals in the three brain regions analyzed (fig. 3b). Similar effects were detected in the relative optical densities of SRC-1 and SRC-2 immunoreactivity between EB- and vehicle-treated animals (data not shown).

Controls for Triple-Label Immunohistochemistry

Controls were performed to confirm specificity of the triple-label immunohistochemistry technique. Omission of each individual primary antibody resulted in no detectable immunoreactivity of the respective label (data not shown). In addition, omission of each individual secondary antibody resulted in no observable immunoreactivity of the respective label (data not shown). Preadsorption of (1) MAB-462 with a 20-fold excess of recombinant human PR-B protein resulted in no PR immunoreactivity, (2) M-20 with a 20-fold molar excess of SRC-1 peptide of the C-terminus resulted in no SRC-1 immunoreactiv-

ity, and (3) NB100-1756 (SRC-2) with a fragment of recombinant human SRC-2 protein resulted in no SRC-2 immunoreactivity (data not shown). In further confirmation of the specificity of the triple-label technique, intensely labeled cells with only PR, SRC-1 or SRC-2 immunoreactivity were observed.

Discussion

Our laboratory and others have shown that the nuclear receptor coactivators SRC-1 and SRC-2 are important for steroid action in the brain. These coactivators modulate ER-mediated transactivation of the PR gene in the brain and ER- and PR-dependent reproductive behaviors in female rodents [54, 61, 64]. In order for nuclear receptor coactivators to function with steroid receptors in brain, both coactivator and receptor must be expressed in the same cell. In the present study, triple-label immunofluorescence was used to investigate the coexpression of E-induced PR with SRC-1 or SRC-2 in individual cells in the female mouse brain.

SRC-1 and SRC-2 were expressed at high levels in the VMN, ARC and MPA, regions known to be involved in reproductive behavior. Interestingly, the majority of E-induced PR cells in the VMN, MPA and ARC coexpress both SRC-1 and SRC-2. Given that virtually all E-induced PR cells in the hypothalamus express ER α [26, 33], the present data indicate that a distinct population of cells in the VMN, MPA and ARC coexpress steroid receptors (ER and PR) and two members of the p160 family of nuclear receptor coactivators (SRC-1 and SRC-2). A smaller population of E-induced PR cells in these three brain regions expressed only SRC-1, while less expressed only SRC-2. Finally, there was a small proportion of E-induced PR cells that expressed neither SRC-1 nor SRC-2. While it is possible that very low levels of SRC-1 and SRC-2 were not detected in PR cells by the immunofluorescent technique, it may be that ER and PR transcriptional activity in these cells is mediated by other coactivators in brain [46]. In future experiments it will be important to investigate other coregulatory proteins that interact with ER and PR, including SRC-3 and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) which have recently been shown to function together to coactivate ER transactivation of the PR gene in MCF-7 cells [82]. It is also possible that the ER and PR in some cells function through a coactivator-independent pathway [83, 84]. It should be noted that not all coactivator-containing cells expressed PR, suggesting that these coactivators function

with other steroid receptors such as glucocorticoid and androgen receptors [46].

There is evidence that SRC-1 and SRC-2 expression in rat brain is altered by steroids [60, 80, 85–88] and endocrine disruptors [89], while other studies have found no effects of steroids on coactivator expression [see 46 for a more thorough review; 48, 49]. However, very little is known about hormonal regulation of coactivators in mouse brain. Therefore, the present study addressed the possibility of E regulation of SRC-1 or SRC-2 expression in mouse brain. EB treatment increased SRC-1 expression in the ARC compared with vehicle-treated control animals. In contrast, EB did not alter SRC-1 levels in the VMN or MPA, or SRC-2 expression in any of the three brain regions. It is possible that the present immunofluorescent technique was not sensitive enough to detect slight changes in SRC-1 or SRC-2 expression in these other brain regions. The present E-induced increase of SRC-1 in the ARC is consistent with other studies in rats that have found changes in SRC-1 protein over the estrous cycle [85] and E-induced increases in SRC-1 mRNA in the hypothalamus [86]. The present findings suggest that E regulates SRC-1, but not SRC-2, in a brain-region-specific manner.

The mechanisms by which individual cells modulate steroid responsiveness in a given brain region is a fundamental issue in steroid hormone action in brain. Taken together with previous findings, the present results identify putative sites of functional interaction of ovarian steroid receptors (ER α and PR) with nuclear receptor coactivators (SRC-1 and SRC-2) in reproductively relevant brain regions. These results support and extend our previous findings that the majority of E-induced PR cells in the rat hypothalamus express SRC-1 and CBP [48] and support the findings that SRC-1 and SRC-2 function in the hypothalamus to modulate hormone-dependent female sexual behavior [54, 61, 64]. In addition, these results provide neuroanatomical support for the concept that these coactivators are important in ER transactivation of the PR gene in brain [54, 61]. However, the functional differences of these coactivators in ER-mediated activation of the PR gene in brain are not known. For example, it is not known if these two coactivators contribute differentially to the ER-mediated induction of the two PR isoforms. In support of this idea, ER and other steroid receptors have distinct affinities for nuclear receptor coactivators. For example, SRC-1 and SRC-2 from the rat hypothalamus and hippocampus physically associate with ER and PR in a receptor subtype- and brain region-specific manner [58, 65]. Interestingly, SRC-1 from the

hypothalamus interacts more with ER α than ER β , while SRC-2 associates with ER α but shows virtually no interaction with ER β [58, 65]. In addition, receptor-coactivator interactions are influenced by different ligands and response elements on DNA [90–93]. In MCS80 Schwann cells, glucocorticoid receptors recruit SRC-1 or SRC-3, but not SRC-2, in the transactivation of a minimal glucocorticoid-sensitive reporter gene containing two GREs [94, 95]. Studies in T47D cells reveal that SRC-1 preferentially recruits CBP resulting in acetylation of histone H4, while SRC-2 recruits pCAF leading to acetylation of histone H3 [96]. It has been suggested that this recruitment of differential histone acetyltransferases can modulate the transcription of various steroid-responsive genes. Moreover, ER (as well as androgen receptors) in the presence of classical hormone response elements, recruit distinct heterodimers of the SRC family members [97]. Interestingly, on non-hormone response element-containing genes (such as AP-1 sites of early genes), ER recruit SRC proteins as monomers. Taken together, the present findings that many E-induced PR cells coexpress both SRC-1 and SRC-2 provide neuroanatomical support for

the concept that steroid responsiveness can be fine-tuned not only by the presence or absence of coactivators but also by the ratio of these coactivators present within individual neurons. Thus, these distinct populations of cells expressing SRC-1 and SRC-2 may allow alternate steroid receptor signaling pathways in the regulation of behavior. Furthermore, the E-induced upregulation of SRC-1 expression in a brain-region-specific manner provides another mechanism by which E can increase the sensitivity of individual cells to steroids. Thus, studying the order and timing of recruitment of different coregulator complexes to the promoter, which is likely to be cell- and region-specific, will be critical to understanding hormone action in the brain.

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