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Steroid Receptor Coactivator-2 Expression in Brain and Physical Associations with Steroid Receptors

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Abstract

Estradiol and progesterone bind to their respective receptors in the hypothalamus and hippocampus to influence a variety of behavioral and physiological functions, including reproduction and cognition. Work from our lab and others has shown that the nuclear receptor coactivators, steroid receptor coactivator-1 (SRC-1) and SRC-2, are essential for efficient estrogen receptor (ER) and progesterin receptor (PR) transcriptional activity in brain and for hormone-dependent behaviors. While the expression of SRC-1 in brain has been studied extensively, little is known about the expression of SRC-2 in brain. In the present studies, we found that SRC-2 was highly expressed throughout the hippocampus, amygdala and hypothalamus, including the medial preoptic area (MPOA), ventral medial nucleus (VMN), arcuate nucleus (ARC), bed nucleus of the stria terminalis, supraoptic nucleus and suprachiasmatic nucleus. In order for coactivators to function with steroid receptors, they must be expressed in the same cells. Indeed, SRC-2 and ER α were coexpressed in many cells in the MPOA, VMN and ARC, all brain regions known to be involved in female reproductive behavior and physiology. While *in vitro* studies indicate that SRC-2 physically associates with ER and PR, very little is known about receptor-coactivator interactions in brain. Therefore, we used pull-down assays to test the hypotheses that SRC-2 from hypothalamic and hippocampal tissue physically associate with ER and PR subtypes in a ligand-dependent manner. SRC-2 from both brain regions interacted with ER α bound to agonist, but not in the absence of ligand or in the presence of the selective ER modulator, tamoxifen. Analysis by mass spectrometry confirmed these ligand-dependent interactions between ER α and SRC-2 from brain. In dramatic contrast, SRC-2 from brain showed little to no interaction with ER β . Interestingly, SRC-2 from both brain regions interacted with PR-B, but not PR-A, in a ligand-dependent manner. Taken together, these findings reveal that SRC-2 is expressed in brain regions

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known to mediate a variety of steroid-dependent functions. Furthermore, SRC-2 is expressed in many ER α containing cells in the hypothalamus. Finally, SRC-2 from brain interacts with ER and PR in a subtype-specific manner, which may contribute to the functional differences of these steroid receptor subtypes in brain.

Keywords

steroid receptor coactivator-2 (SRC-2); estrogen receptor; progesterin receptor; hypothalamus; hippocampus; reproductive behavior

INTRODUCTION

The ovarian steroid hormones, estradiol and progesterone, act in the brain to profoundly influence a variety of physiological and behavioral events, including development, cognition, and reproduction (Blaustein and Mani, 2006; Pfaff et al., 2009). Estradiol and progesterone elicit many of these biological effects by binding to receptors for estrogens (ER) and progestins (PR), respectively, in specific brain regions. ER and PR are members of the steroid/nuclear receptor superfamily of transcriptional activators (Mangelsdorf et al., 1995). In brain, these receptors act in a classic, genomic mechanism by interacting directly with DNA to regulate gene transcription. In addition, steroid receptors in brain can function independent of ligand on either DNA or at the membrane to rapidly activate cytoplasmic signaling pathways (Olesen et al., 2005; Kelly and Ronnekleiv, 2008; Micevych and Mermelstein, 2008; Vasudevan and Pfaff, 2008; Mani et al., 2009; Tetel and Lange, 2009).

Intracellular ER exist as two subtypes, α and β , which are transcribed from different genes (Jensen et al., 1968; Shyamala and Gorski, 1969; Kuiper et al., 1996). These two ER subtypes differ in their abilities to bind ligands (Kuiper et al., 1997; Hall and McDonnell, 1999; Jones et al., 1999; Dandimopoulos et al., 2008), distribution in brain (Shughrue et al., 1997; Osterlund et al., 1998; Greco et al., 2001; Mitra et al., 2003), and function in brain and behavior (Ogawa et al., 1998; Ogawa et al., 1999; Bodo and Rissman, 2006; Musatov et al., 2006). Furthermore, cell culture experiments indicate that ER α is a stronger transcriptional activator than ER β due to differences in the AF-1 region (Delaunay et al., 2000). 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 (Kastner et al., 1990). Under certain cell and promoter contexts, PR-B is a stronger transcriptional activator than PR-A (Giangrande et al., 1997; Tung et al., 2006) due to an additional activation function in the up-stream sequences unique to PR-B (Sartorius et al., 1994; Wen et al., 1994). Studies using isoform specific knockout mice reveal that PR-A and PR-B have distinct functions in reproductive behavior and physiology (Mulac-Jericevic and Conneely, 2004; Mani et al., 2006).

Efficient steroid receptor transcription requires a class of proteins known as nuclear receptor coregulators, which consist of coactivators and corepressors. These coregulators play an important role in a variety of human diseases, including cancer and some neurological disorders (Lonard et al., 2007). Nuclear receptor coactivators are rate-limiting in steroid receptor-mediated gene transcription (Oñate et al., 1995; O'Malley, 2006; Rosenfeld et al., 2006). The importance of coactivators is further evident in their ability to reverse the squelching of the transcriptional activity of one steroid receptor by another (Oñate et al., 1995). In addition to functioning as a bridge between receptors and the general transcriptional machinery, nuclear receptor coactivators influence receptor transcription through a variety of mechanisms, including phosphorylation, acetylation, methylation, RNA splicing and chromatin remodeling (Lonard and O'Malley, 2006; Rosenfeld et al., 2006).

The p160 steroid receptor coactivator (SRC) family includes SRC-1/NcoA-1 (Oñate et al., 1995); SRC-2/TIF2/GRIP1/NcoA2 (Voegel et al., 1996; Hong et al., 1997); and SRC-3/pCIP/ACTR/AIB1/TRAM-1/RAC3 (Anzick et al., 1997; Suen et al., 1998). These coactivators dramatically enhance the transcriptional activity of a variety of steroid receptors, including ER and PR (Oñate et al., 1995; O'Malley, 2006; Rosenfeld et al., 2006). This p160 SRC family of coactivators, along with other coactivators, is thought to increase transcriptional activity through a variety of mechanisms, including acetylation of histones, methylation, phosphorylation and chromatin remodeling (O'Malley, 2006; Rosenfeld et al., 2006). *In vitro* studies indicate that under most conditions, ER and PR interact with the SRCs in the presence of an agonist, but not in the absence of ligand or in the presence of an antagonist or a selective receptor modulator (Oñate et al., 1995; McInerney et al., 1996; Shiau et al., 1998; Tanenbaum et al., 1998) but *c.f.* with (Oñate et al., 1998; Webb et al., 1998; Dutertre and Smith, 2003). Selective ER modulators (SERMs, *e.g.* tamoxifen) and selective PR modulators (SPRMs, *e.g.* RU486) regulate ER and PR activity, respectively, in a tissue-specific manner (Lewis-Wambi and Jordan, 2005; Wardell and Edwards, 2005; Han et al., 2007). Whether these receptor modulators block or activate receptor action appears to be dependent on the cellular environment, including the ratio of coactivators and corepressors (Smith et al., 1997).

While SRC-2 shares some sequence homology with the other two members of the p160 coactivator family, distinct physiological functions of SRC-2 have been identified. SRC-2 knock-out mice reveal that this coactivator is important in fertility and ductal branching in mammary gland (Gehin et al., 2002; Fernandez-Valdivia et al., 2007; Mukherjee et al., 2007). Generation of mice in which SRC-2 is ablated specifically in cell types that express PR (PR^{Cre/+}SRC-2^{flox/flox}) has revealed that SRC-2 functions in progesterin-dependent embryo implantation (Fernandez-Valdivia et al., 2007). Microarray analysis of uteri from SRC-2 null mice reveal that this coactivator is critical for the ability of progesterone to repress specific genes involved in a variety of functions, including cell cycle and immunity (Jeong et al., 2007). SRC-2 also functions to regulate glucose production (Chopra et al., 2008) and bone mass (Modder et al., 2009). Finally, SRC-2 appears to be involved in ER α regulated cell proliferation of breast cancer cells (Karmakar et al., 2009; Xu et al., 2009).

Studies from our lab and others reveal that nuclear receptor coactivators function in hormone action in the central and peripheral nervous systems (Tetel, 2009; Tetel et al., 2009). For example, SRC-1 is expressed in cells containing estradiol-induced PR (Tetel et al., 2007) and is important for hormone-dependent sexual differentiation of the brain (Auger et al., 2000), gene expression in brain (Apostolakis et al., 2002; Molenda et al., 2002; Charlier et al., 2005; Charlier et al., 2006) and sexual behavior (Apostolakis et al., 2002; Molenda et al., 2002; Charlier et al., 2005; Charlier et al., 2006; Molenda-Figueira et al., 2006). While the function of SRC-1 in brain has been well-studied, less is known about the role of SRC-2 in brain. While no detailed neuroanatomical studies have been reported, *in situ* hybridization and Western blot analyses reveal SRC-2 is expressed at high levels in the hippocampus, cerebellum and hypothalamus (Apostolakis et al., 2002; Nishihara et al., 2003; McGinnis et al., 2007). Moreover, in females SRC-2 is important in hormone-dependent lordosis and estradiol-induction of PR in the hypothalamus (Apostolakis et al., 2002). Finally, SRC-2, as well as SRC-1, function in glucocorticoid receptor mediated gene expression in astrocytes (Grenier et al., 2006; van der Laan et al., 2008).

A variety of cell culture studies indicate that receptor-coactivator interactions occur in a ligand-dependent manner (Oñate et al., 1995; McInerney et al., 1996; Shiau et al., 1998; Tanenbaum et al., 1998). Recent work from our lab reveals that SRC-1 from brain physically interacts with ER and PR in a receptor subtype-specific and ligand-dependent manner (Molenda-Figueira et al., 2008). However, it is not known if SRC-2 from brain

physically associates with steroid receptors. In the present studies, we investigated the expression pattern of SRC-2 in the female rat hypothalamus and asked if SRC-2 and ER α were coexpressed in cells from hypothalamic regions known to regulate female reproduction. Furthermore, using pull-down assays, we tested the hypotheses that SRC-2 from brain regions rich in steroid receptors physically associate with ER and PR subtypes in a ligand-dependent manner.

EXPERIMENTAL PROCEDURES

Experimental animals

Adult female (175–200g) Sprague-Dawley rats from Charles River Laboratories, Inc. (Wilmington, MA) were housed four animals to a cage in a 14:10 light/dark cycle with lights off at 11 a.m. Animals were given food and water *ad libitum*. Female rats were anesthetized with Ketamine/Xylazine cocktail (100 mg Ketamine and 18 mg xylazine/0.75 ml/kg in saline) and ovariectomized. A one-week recovery period followed to allow clearing of endogenous hormones. All animal procedures were approved by the Institutional Animal Care and Use Committees of Skidmore College and Wellesley College.

Immunohistochemical analysis of SRC-2 expression in brain

For immunohistochemical studies, animals (n=6) were overdosed with sodium pentobarbital (89 mg/kg) and chloral hydrate (425 mg/kg) and perfused with 4% paraformaldehyde. Five thousand units of sodium heparin dissolved in 1 ml of saline were injected into the left ventricle. Saline (0.15 M, 25 ml) preceded the flow of 250 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH=7.2) at a flow rate of 25 ml/min for 10 minutes. 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 overnight. Forty μ m sections were cut through the hypothalamus on a freezing rotary microtome and stored in cryoprotectant at -20°C until immunohistochemistry.

Single-label Immunohistochemistry for SRC-2—Sections were initially rinsed in 0.05M Tris-buffered saline (TBS). Tissue was then rinsed in TBS and incubated in a solution of 1% H₂O₂, 20% normal goat serum and 1% bovine serum albumin in TBS for 20 minutes to decrease nonspecific staining and reduce endogenous peroxidase activity. Sections were incubated for 48 hours with a mouse monoclonal antibody generated against amino acids 959–1067 of human SRC-2 (TIF 2, 1.9 μ g/ml, BD Trans Lab) in TBS containing 0.02% sodium azide (NaN₃), 1% normal goat serum, 0.1% gelatin and Triton X-100 (pH 7.6 at 4°C). After rinsing, the tissue was incubated for 90 minutes in biotinylated goat-anti-mouse secondary antibody (3 μ g/ml, Jackson Laboratory, West Grove, PA) containing NaN₃ and Triton X-100 and 1.5% normal goat serum. Tissue was rinsed in TBS containing NaN₃, gelatin and Triton X-100 followed by rinsing in TBS. Sections were then incubated for 90 minutes in TBS containing 1% avidin DH: biotinylated horseradish peroxidase H complex (Vectastain ABC Elite Kit, Vector, Burlingame, CA) followed by rinsing in TBS. Finally, sections were exposed to 0.05% diaminobenzine (DAB) with 3% hydrogen peroxide with TBS for approximately 10 minutes. The sections were rinsed in TBS and then mounted on microscope slides and coverslipped using DePeX mounting medium (Electron Microscopy Sciences, PA). One matched section for each brain area from the hypothalamus, hippocampus and amygdala (Paxinos and Watson, 1998) were investigated. SRC-2 immunoreactive cells were visualized under 100x magnification using an Olympus BX60 microscope.

Dual-label Immunofluorescence for ER α and SRC-2—Brain sections were mounted onto subbed slides, dried and then washed three times in PBS for 5 min. Tissue was

permeabilized with 1% SDS (ACROS), 8% betamercaptoethanol (Sigma) and 4% normal goat serum (PelFreeze, Rogers, AR) in PBS. To detect ER α and SRC-2, sections were incubated in a cocktail containing a rabbit polyclonal antibody generated against the last 15 amino acids of rat ER α (1:15,000, C1355, Upstate) and the SRC-2 monoclonal, TIF2 (1.9 ug/ml), in TBS at 4°C overnight. Sections were washed with PBS and then incubated in 1% BSA and 4% normal goat serum in PBS for 30 min. Sections were incubated for one hour in a cocktail of fluorescently-labeled secondary antisera containing CY3-labeled goat anti-rabbit serum (1ug/ml, Jackson ImmunoResearch) for visualization of ER α and FITC-labeled goat anti-mouse serum (4 ug/ml, Jackson ImmunoResearch) for detection of SRC-2. Sections were washed in PBS, dried and slides were cover-slipped with Vectashield mounting medium (Vector Laboratories) diluted 1:1 with 0.3M Tris (pH 8.8). Images of immunofluorescence from the left side of one matched section per brain region for each rat (Paxinos and Watson, 1998) were captured at 200X using an Olympus Fluoview FV300 confocal system equipped with Argon and He-Ne lasers. Images of one optical section at a thickness of 1 μ m were taken at the top of each brain section within a consistent region of interest for all animals per brain region and converted to tif files for analysis. Controls for the immunohistochemistry included the omission of the primary or secondary antibodies.

Recombinant flag and GST-tagged steroid receptors

Recombinant ER and PR fusion proteins were expressed in *Spodoptera frugiperda* (Sf9) insect cells by the Baculovirus/Monoclonal Antibody Facility of the Baylor College of Medicine as described previously (Tetel et al., 1999; Melvin et al., 2004). Full-length human ER α or ER β were fused to a flag tag (viruses kindly provided by Lee Kraus, Cornell Univ.) (Kraus and Kadonaga, 1998; Melvin et al., 2004). Sf9 cell cultures for ER-flag were incubated with saturating doses of 200 nM estradiol, 200 nM 4-OH-tamoxifen, or no ligand. Full-length human PR-A or PR-B was fused to a glutathione *S*-transferase (GST) tag. Insect cell cultures for PR-GST (viruses kindly provided by David Bain, Univ. Colorado HSC) were incubated with saturating doses of 200 nM of the PR agonist R5020, 200 nM of the SPRM RU486, or in the absence of PR ligand. Sf9 cell pellets were homogenized in homogenization buffer (10 mM Tris, 10% glycerol, 400 mM NaCl, 1 mM DTT, 1mM EDTA, pH = 7.4) with protease inhibitors (1:10 dilution, P2714, Sigma, Saint Louis, MO). Samples were incubated on ice for 30 min, and then centrifuged for 30 min at 4°C at 40,000 rpm and supernatants were stored at -80°C.

Tissue preparation

Ovariectomized rats were overdosed with sodium pentobarbital (89 mg/kg) and chloral hydrate (425 mg/kg) and then decapitated. Hypothalamic and hippocampal (containing a small portion of the cortex dorsal to the hippocampus) tissues were dissected out, flash frozen on dry ice and stored at -80°C. Brain tissue from female rats (n=54) was pooled in groups of three for each sample and homogenized in buffer (10 mM Tris, 10% glycerol, 400 mM NaCl, 1 mM DTT, 1mM EDTA, pH = 7.4) with protease inhibitors (1:10 dilution, P2714, Sigma). Samples were incubated on ice for 30 min., and then centrifuged for 30 min. at 4°C at 12,000 rpm and supernatants were aliquotted and frozen at -80°C.

ER flag-tagged pull-down

ER flag-tagged pull down assays were conducted at 4 C as described previously (Molenda-Figueira et al., 2008). Briefly, twenty-five microliters of packed Anti-flag M2 affinity gel resin (Sigma) were added to each siliconized centrifuge tube and pre-washed 3 times with TBS and 2 times with 100 mM glycine HCl (pH = 3.5). Resins were next washed 3 times with Wash Buffer + NaCl (50mM Tris-HCl, 100mM NaCl, 1% glycerol, 50mM Na Fluoride, pH = 7.4) + TX-100 (0.1% Triton X-100). Equal amounts of recombinant flag-tagged ER were added to the resin column and rotated on an end-over-end rotator for 1 hour.

The resins, with immobilized ER, were washed 3 times with Wash Buffer + NaCl. Equal amounts of pooled hypothalamic or hippocampal whole cell extracts were added to the immobilized ER-flag and incubated on a rotator for 1 hour. The resins were washed 3 times with Wash Buffer + NaCl to eliminate non-specific binding, and then samples were eluted with 2% SDS sample buffer by boiling samples for 5 minutes and stored at -80°C until use.

Samples were analyzed by Western blot as described previously (Molenda-Figueira et al., 2006) for detection of SRC-2 interactions with ER. Blots were probed for SRC-2 from brain by incubating overnight with the mouse monoclonal antibody described above (TIF 2, 1.0 $\mu\text{g}/\text{ml}$, BD Trans Lab). Membranes were washed in TBS-T and incubated in a sheep anti-mouse antibody (1:6000, Amersham Biosciences, Uppsala, Sweden). Following washes in TBS-T, immunoreactive bands were detected with an enhanced chemiluminescence kit (ECL; New England Biolabs, Ipswich, MA), and the membranes were scanned using a PhosphorImager (STORM Scanner 860, Molecular Dynamics) and exposed to film (Blue Sensitive X-ray film, Laboratory Products Sales, Rochester, NY). Images from the PhosphorImager were imported into the ImageQuant analysis program (V.5.2, Molecular Dynamics) and analyzed for integrated density (area of band \times mean optical density) of immunoreactive bands. Membranes were stripped for 2 hours at 70°C in stripping buffer (2% sodium laurel sulfate, 62.5 mM Tris HCl, 100 mM 2-mercaptoethanol, H_2O , pH = 6.7) and re-probed for flag-tagged ER using a mouse monoclonal antibody generated against the flag-tag (0.5 $\mu\text{g}/\text{ml}$, anti-Flag M2, Sigma) and a sheep anti-mouse secondary antibody (1:80,000 dilution, Amersham Biosciences). Immunoreactive bands were analyzed as described above.

PR-GST pull-down

PR-GST pull down assays were done as described previously (Molenda-Figueira et al., 2008). Fifty microliters of Glutathione Sepharose 4B packed resins (0.05 $\mu\text{g}/\mu\text{l}$, Amersham Biosciences) were added to siliconized centrifuge tubes and pre-treated with ovalbumin (1 mg/ml, Fisher Scientific, Hampton, NH) for 15 minutes on an end-over-end rotator and rinsed three times with TG buffer (20 mM Tris-HCl, 10% glycerol; pH 8.0) containing 100 mM NaCl (TG + NaCl). Equal amounts of recombinant human PR-GST suspended in TG buffer were added to resins and incubated on a rotator for 1 hour. Following the incubation, the resins with immobilized PR-GST were washed four times with TG + NaCl. Equal amounts of pooled hypothalamic or hippocampal whole cell extracts were added to immobilized PR-GST and incubated on a rotator for 1 hour. The resins were washed four times with TG + NaCl.

Samples were eluted in 2% SDS sample buffer as described above and stored at -80°C until analysis. Samples were analyzed by Western blot, as described above, to detect SRC-2 interacting with PR. SRC-2 immunoreactive bands were detected using a PhosphorImager and analyzed as described above (STORM Scanner 860, Molecular Dynamics). Membranes were stripped and probed for PR-A and PR-B, using a mouse monoclonal antibody that recognizes the N-terminal amino acids 165–534 of both PR-A and PR-B (PR 1294, 0.1 $\mu\text{g}/\text{ml}$, kindly provided by Dean Edwards, Baylor College of Medicine), followed by a sheep anti-mouse secondary antibody (1:10,000, Amersham). PR-immunoreactive bands were analyzed as described above.

Mass Spectrometry

Rat hypothalamic extracts (approximately 40 mg of tissue per condition) were exposed to immobilized ER α in the presence of 200 nM estradiol or no ligand. Eluted samples were resolved in adjacent lanes by SDS-PAGE and the region of the gel corresponding to SRC-2 was excised, digested with trypsin and desalted as described previously (Zhao et al., 2003;

Tilton et al., 2007). The peptide mixture was injected onto a C18 trap and then separated on a reversed phase nano-HPLC column (PicoFrit™, 75 $\mu\text{m} \times 10 \text{ cm}$; tip ID 15 μm) with a linear gradient of 0–50 % mobile phase B (0.1 % formic acid–90 % acetonitrile) in mobile phase A (0.1 % formic acid) over 120 min at 200 nl/min. LC-MS/MS experiments were performed with a LTQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a nanospray source; the mass spectrometer was coupled on-line to a ProteomX® nano-HPLC system (ThermoFinnigan, San Jose, CA). The mass spectrometer was operated in the data-dependent mode using XCalibur software. The most intense seven ions in each MS survey scan were automatically selected for MS/MS. This approach allows the detection of individual proteins in the nanogram range and has been used to identify SRC-1 in multiprotein complexes using immunoaffinity purification as well as low abundance transcription factors such as RelA/p65 NFkB (Zhao et al., 2003; Tilton et al., 2007). The acquired MS/MS spectra were searched with SEQUEST algorithm from the SWISSPROT Protein Database on the Bioworks 3.2 platform (ThermoFinnigan, San Jose, CA).

Statistical analysis

The amount of SRC-2 in each pull-down sample was normalized to the amount of SRC-2 in the input whole cell extract by creating a ratio of the integrated density of the SRC-2 immunoreactive band to the integrated density of input of SRC-2 for the experiment. Unless stated otherwise, the integrated density of immunoreactive bands was analyzed using a two-way ANOVA (SPSS) to determine differences between receptor subtypes and ligand conditions. A Tukey's HSD test was used for *post-hoc* comparisons. Differences were considered significant at a probability of less than 0.05.

RESULTS

SRC-2 and ER α are coexpressed in individual cells in brain

SRC-2 immunoreactivity (SRC2-IR) was observed at high levels in the female rat ventral medial nucleus (VMN), arcuate nucleus (ARC), posterodorsal medial amygdala (MePD), medial preoptic area (MPOA) and supraoptic nucleus (Figure 1), as well as the bed nucleus of the stria terminalis (BNST), suprachiasmatic nucleus and hippocampus. Moderate to lower levels of SRC2-IR were detected in the habenular and paraventricular nuclei. These findings are consistent with, and extend, previous work revealing SRC-2 expression in the hypothalamus and hippocampus of rats (Apostolakis et al., 2002; McGinnis et al., 2007) and mice (Nishihara et al., 2003).

Consistent with previous studies, we found ER α -IR cells in the same regions of the preoptic area, hypothalamus and amygdala as described previously in rats using [^3H]autoradiography, *in situ* hybridization and immunohistochemistry (Stumpf and Sar, 1971; Pfaff and Keiner, 1973; Cintra et al., 1986; Lauber et al., 1990; Simerly et al., 1990; Blaustein, 1992; Tetel et al., 1994). Moreover, many ER α -IR cells also expressed SRC-2 in the VMN and ARC (Figure 2), as well as the MPOA and BNST. In addition, there were ER α -IR cells that did not express SRC-2 and SRC2-IR cells that lacked ER α . Omission of the primary monoclonal antibody for SRC-2 from the immunohistochemical procedure resulted in no detectable SRC-2 immunoreactive cells in any brain region. Omission of the primary antibody for ER from the immunohistochemical procedure resulted in no ER-IR cells. In further confirmation of the specificity of the double label immunofluorescent technique, as stated above, intensely labeled ER-IR cells devoid of SRC-2 were observed, as well as SRC2-IR cells that lacked ER-IR.

SRC-2 from brain associates more with agonist-bound ER α than with ER β

ER-flag tag pull-down assays were performed to determine whether SRC-2 interacts with ER α or ER β and whether these associations were ligand-dependent. Western blot analysis of pull-down assays revealed that SRC-2 from female rat hypothalamus interacted with ER α in the presence of the agonist, estradiol, while little interaction was detected when ER α was not bound to ligand or bound to the SERM, tamoxifen (Figure 3B). Quantification of these ligand-dependent interactions between SRC-2 from the hypothalamus and ER α are shown in Figure 3D. As in the hypothalamus, SRC-2 from the hippocampus physically interacted with ER α in the presence of estradiol, but not when unbound or bound to tamoxifen (Figures 3A & C; $F(2, 24) = 37.06, P < 0.0001$). These findings indicate that SRC-2 from hypothalamus or hippocampus interacts with ER α in a ligand-dependent manner.

In dramatic contrast to the high levels of association between SRC-2 and estradiol-bound ER α , little interaction was detected between agonist-bound ER β and SRC-2 from brain (Figure 3). SRC-2 from the hypothalamus interacted with ER β in a ligand-dependent manner to some extent (Figure 4B; $F(2, 9) = 6.004, P < 0.03$), while there was a trend towards association of SRC-2 from hippocampus with ER β bound to estradiol (Figure 3C & D). Moreover, SRC-2 from either brain region interacted more with estradiol-bound ER α than ER β (hippocampus: $F(1, 24) = 27.47, P < 0.0001$; hypothalamus: $F(1, 18) = 13.58, P < 0.003$). These findings suggest that SRC-2 physically associates with ER in a receptor subtype specific manner.

Mass spectrometry confirms the ligand-dependent interactions of hypothalamic SRC-2 with ER α

In order to independently confirm the western blot data for estradiol-dependent binding of ER α to SRC-2 from rat brain, we employed an unbiased mass spectrometry approach. Rat hypothalamic extracts were exposed to immobilized ER α in the presence of estradiol or no ligand and eluted samples were resolved by SDS-PAGE. Gel slices corresponding to the putative SRC-2 region of the 2 lanes were digested with trypsin, and peptides analyzed by LC-MS/MS. Table 1 shows that database searching identified three highly significant, doubly charged peptides corresponding uniquely to rat SRC-2 (UniProt Accession # Q9WUI9) in the gel slice from the estradiol treated sample. All three peptides had XCorr values 3.5 and Cn 0.40 with excellent identification of fragments ions. These peptides corresponded to amino acids 100–112, (SDVSSTGQGVIDK), 693–705 (LLQDSSSPVDLAK), and 714–731 (ELNQESSGTAPGSEVTVK). In confirmation of the western blot analyses, no SRC-2 peptides were identified in the sample lane from unliganded ER α .

PR interacts with neural SRC-2 in a receptor subtype-specific manner

PR-GST pull-down assays were done to determine if SRC-2 from rat brain physically interacts with PR-A or PR-B, and if these interactions occur in a ligand-dependent manner. Western blots from GST-tag pull-down assays indicated that SRC-2 from hippocampus interacted with PR-B in the presence of the agonist R5020, but little to no interactions were detected with PR-B in the absence of ligand or in the presence of the SPRM, RU486 (Figure 4A & C; $F(2, 40) = 9.19, P < 0.002$). Similar findings were made with SRC-2 from the hypothalamus, suggesting that SRC-2 from these brain regions interact with PR-B in a ligand-dependent manner (Figure 4B & D; $F(2, 39) = 9.20, P < 0.002$). While the ligand-dependent interactions of SRC-2 from brain with PR-A did not reach significance, there was a trend towards these interactions occurring in a ligand-dependent manner (Figure 4C & D).

DISCUSSION

In vitro studies indicate that SRC-2 interacts with ER and PR, and dramatically enhances the transcriptional activity of these steroid receptors (Voegel et al., 1996; Hong et al., 1997). While the function of SRC-1 in hormone action in brain and behavior has been studied in some depth (Tetel, 2009; Tetel et al., 2009), the role of SRC-2 in brain function has not been well-defined. Thus, the present study investigated if SRC-2 was expressed in steroid-sensitive brain regions. In order for SRC-2 to function with steroid receptors, SRC-2 must be expressed in receptor-containing cells. Therefore, we asked if individual cells coexpressed SRC-2 and ER α . Finally, we tested the hypothesis that SRC-2 from brain regions rich in steroid receptors physically associates with ER and PR subtypes in a ligand-dependent manner.

Previous analyses by Western blots have shown that SRC-2 is expressed in the rat hypothalamus (Apostolakis et al., 2002; McGinnis et al., 2007). The present studies used immunohistochemistry to extend these findings to reveal that SRC-2 is expressed at high levels in the female rat MPOA, VMN, ARC, posterodorsal medial amygdala, bed nucleus of the stria terminalis, supraoptic nucleus, suprachiasmatic nucleus and hippocampus. In addition, moderate to lower levels of SRC-2 were detected in the habenular and paraventricular nucleus. Moreover, many cells in the MPOA, VMN and ARC coexpressed ER α and SRC-2. These results extend our previous findings that other nuclear receptor coactivators, SRC-1 and CBP, are expressed in ER α -containing cells in the female hypothalamus (Tetel et al., 2007). In addition, many ER α containing cells in these brain regions also express PR (Blaustein and Turcotte, 1989; Warembourg et al., 1989), suggesting that a subpopulation of the coexpressing cells identified in the present study also express PR. While not all ER α -containing cells expressed SRC-2, it may be that the immunohistochemical technique was not sensitive enough to detect low levels of SRC-2. Alternatively, it is possible that this sub-population of ER α -expressing cells is regulated by other coactivators. In addition, there were SRC2-IR cells that did not express ER α , suggesting that SRC-2 may function with other receptors, including those for androgens (AR) and glucocorticoids (GR), in these cells (Voegel et al., 1996; Hong et al., 1997). In future studies, it will be important to determine if SRC-2 is coexpressed with other steroid receptors, including ER β , PR, AR and GR. The present findings provide neuroanatomical evidence suggesting that SRC-2 functions in ER α action in brain regions discussed above that are known to be involved in a variety of functions, including reproduction (Pfaff et al., 2009), stress (Krishnan and Nestler, 2008), circadian rhythms (Silver et al., 1996; Hastings et al., 2008), metabolism (King, 2006; Musatov et al., 2007) and cognition (Fugger et al., 2000; Bodo and Rissman, 2006).

We also tested the hypothesis that SRC-2 from female rat hippocampus and hypothalamus, regions rich in ER and PR (Pfaff and Keiner, 1973; MacLusky and McEwen, 1978; Blaustein et al., 1988)(Shughrue et al., 1997; Osterlund et al., 1998; Greco et al., 2001; Mitra et al., 2003), physically associates with ER and PR subtypes in a ligand-dependent manner. Using pull-down assays with brain tissue, we found that SRC-2 from hypothalamic and hippocampal extracts interacted with flag-tagged ER α when bound to the agonist estradiol. In contrast, little to no interactions were observed between SRC-2 and ER α in the absence of ligand or in the presence of tamoxifen, indicating that this SERM is acting as an antagonist in our assays. Analysis by mass spectrometry confirmed that SRC-2 from rat brain interacted with ER α in a ligand-dependent manner. These results are consistent with *in vitro* studies that show SRC-2 interacts with ER α in the presence of agonist (Voegel et al., 1996; Hong et al., 1997; Webb et al., 1998; Privalsky et al., 2009). In addition, these findings support previous studies that SRC-2 acts in the hypothalamus to modulate estrogen

action (Apostolakis et al., 2002) and suggest that SRC-2 functions with ER α in cognition in the hippocampus (Fugger et al., 2000; Bodo and Rissman, 2006).

In contrast to ER α , ER β did not interact with SRC-2 from hippocampus under any ligand condition, including when bound to estradiol. In addition, ER β bound to estradiol interacted very weakly with SRC-2 from hypothalamus. This weak interaction between agonist-bound ER β and SRC-2 from brain is in contrast to cell culture studies indicating over-expressed SRC-2 does interact with ER β (Kraichely et al., 2000; Routledge et al., 2000; Zhao et al., 2005; Cvoro et al., 2008). One explanation for the differences between the present results using SRC-2 from rat brain and other studies using recombinant SRC-2 (Kraichely et al., 2000) may be that over-expression of coactivators leads to altered interactions with receptors. In addition, the presence of other factors in brain that may mediate appropriate receptor-coactivator associations point to the significance of using biologically-relevant tissue in studying these important interactions. Finally, cell culture studies suggest that both ER α and ER β can recruit SRC-2 and other coactivators in the absence of ligand under certain phosphorylation conditions (Webb et al., 1998; Yi et al., 2002; Bai and Giguere, 2003). While we detected little to no interactions between ER and SRC-2 from brain in the absence of ligand, it will be important to investigate whether physiologically-relevant events that modulate ligand-independent activation can influence receptor-coactivator interactions in brain.

Both ER α and ER β are expressed in the hypothalamus (Shughrue et al., 1997; Kuiper et al., 1998; Osterlund et al., 1998; Greco et al., 2001; Mitra et al., 2003). Hypothalamic ER α are necessary for the full expression of female sexual behavior (Rissman et al., 1997; Ogawa et al., 1998; Ogawa et al., 1999; Kudwa and Rissman, 2003; Musatov et al., 2006), while expression of ER β in the hypothalamus seems to be more important in anxiety and the stress response (Isgor et al., 2003; Imwalle et al., 2005; Lund et al., 2005; Bodo and Rissman, 2006). These dramatic differential functions of the ER subtypes in brain and behavior may be explained in part by the differential interactions of these receptor subtypes with coactivators reported here. In addition, in some cell lines, ER α is a stronger transcriptional activator than ER β (Delaunay et al., 2000). Thus, the present findings that SRC-2 from brain interacts strongly with ER α , but not ER β , provide a possible mechanism for the functional differences of these ER subtypes.

Our results also show that SRC-2 from rat hippocampus or hypothalamus physically interact with PR-B in a ligand-dependent manner. SRC-2 from both of these brain regions associated more with PR-B in the presence of the agonist, R5020, than in the absence of ligand or in the presence of the SPRM, RU486. In contrast, while there was a trend towards ligand-dependent interactions of SRC-2 from the hippocampus or hypothalamus with PR-A, statistical significance was not reached, suggesting PR isoform specific interactions with SRC-2. These differential interactions between SRC-2 and the PR isoforms are supported by some cell culture studies (Giangrande et al., 2000), while other studies indicate that PR-A can interact with SRC-2 under certain promoter contexts (Heneghan et al., 2007). Cell culture studies suggest that under certain circumstances, PR-B is a stronger transcriptional activator than PR-A (Giangrande et al., 1997; Giangrande et al., 2000; Tung et al., 2006), likely due to the additional activation function (AF-3) of PR-B (Sartorius et al., 1994; Wen et al., 1994). Moreover, studies using PR-A and PR-B specific knock-outs reveal that both receptors are important for the full display of progesterone-facilitated lordosis (Mani et al., 2006). Interestingly, PR-A has a greater role than PR-B in ligand-independent lordosis facilitated by the cyclic AMP analogue, 8-bromo-cAMP (Mani et al., 2006). In future studies, it will be important to investigate the function of SRC-2 and other coactivators in ligand-independent activation of PR.

As discussed in our previous work (Molenda-Figueira et al., 2008), it should be noted that human ER and PR proteins were used to detect interactions with rat SRC-2 from brain. Therefore, we cannot exclude the possibility that SRC-2 from rat brain may interact differently with rat ER or PR compared with human. However, the ligand binding domains (LBDs) of human ER (α and β) and PR, the most important receptor region for mediating SRC-2 interactions (Heery et al., 1997; Feng et al., 1998), have highly homologous protein sequences (89–92% identical) with the LBDs of rat ER and PR, respectively (Kastner et al., 1990; Kato et al., 1993; Harris et al., 2002). Given that protein-protein interactions are sensitive to protein structure, in future studies it will be important to study the interactions of rat SRC-2 with rat ER and PR using other approaches such as coimmunoprecipitation assays. Even so, the high degree of homology between rat and human LBDs of ER and PR, the ligand dependency of the interactions detected in the present studies, and the confirmation by mass spectrometry, indicate that these results offer important information about the interactions between ER and PR with SRC-2 from brain.

In summary, these findings indicate that SRC-2 is expressed in steroid sensitive regions in the hypothalamus. Moreover, a subpopulation of hypothalamic cells coexpress SRC-2 and ER, extending our previous work showing the coexpression of other coactivators (SRC-1 and CBP) with steroid receptors in brain (Tetel et al., 2007). In addition, using pull-down assays, we found that SRC-2 from hypothalamus and hippocampus interact differently with the ER and PR subtypes. These results support previous work showing that SRC-2 is important for ER-mediated induction of PR in brain and the expression of hormone-dependent female sexual behavior (Apostolakis et al., 2002). Presumably, these interactions between SRC-2 and receptors are important for reproductive development in males and females (Gehin et al., 2002; Fernandez-Valdivia et al., 2007; Mukherjee et al., 2007), pregnancy (Gehin et al., 2002), hormone-dependent gene transcription in brain (Apostolakis et al., 2002) and female sexual behavior (Apostolakis et al., 2002). In addition, the present findings reveal the importance of using biologically-relevant brain tissue when studying these interactions. Finally, estrogens and progestins are involved in a variety of human diseases. For example, estrogen therapy may reduce the risk of Alzheimer's disease (Pike et al., 2009). Interestingly, expression in brain of an ER α mutant, that may prevent coactivator binding, has been correlated with Alzheimer's disease (Ishunina and Swaab, 2009). In future studies, it will be critical to determine the functional significance of the differential interactions of SRC-2 with the receptor subtypes on gene expression in brain and the potential role in disease.

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Abbreviations

AR	androgen receptor
ARC	arcuate nucleus
BNST	bed nucleus of the stria terminalis
CBP	CREB binding protein
ER	estrogen receptor
GR	glucocorticoid receptor

GST	glutathione-S-transferase
IR	immunoreactivity
LBD	ligand binding domain
MePD	posterodorsal medial amygdala
MPOA	medial preoptic area
PR	progesterin receptor
SERM	selective estrogen receptor modulator
SPRM	selective progesterin receptor modulator
SRC-1	steroid receptor coactivator-1
SRC-2	steroid receptor coactivator-2
VMN	ventral medial nucleus of the hypothalamus

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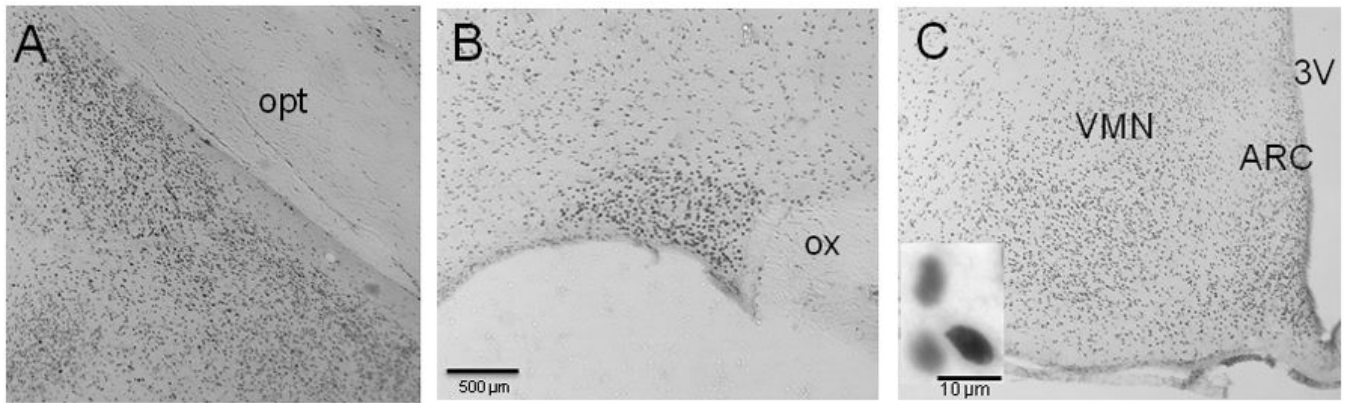


Figure 1. SRC-2 immunoreactive cells in the A) posterodorsal portion of the medial amygdala (MePD), B) supraoptic nucleus (SON), magnification bar = 500 μm, and C) ventromedial nucleus of the hypothalamus (VMN) and arcuate nucleus (ARC) of the female rat. Inset shows nuclear immunostaining of cells from the VMN, magnification bar = 10 μm. opt = optic tract; ox = optic chiasm; 3V = third ventricle.

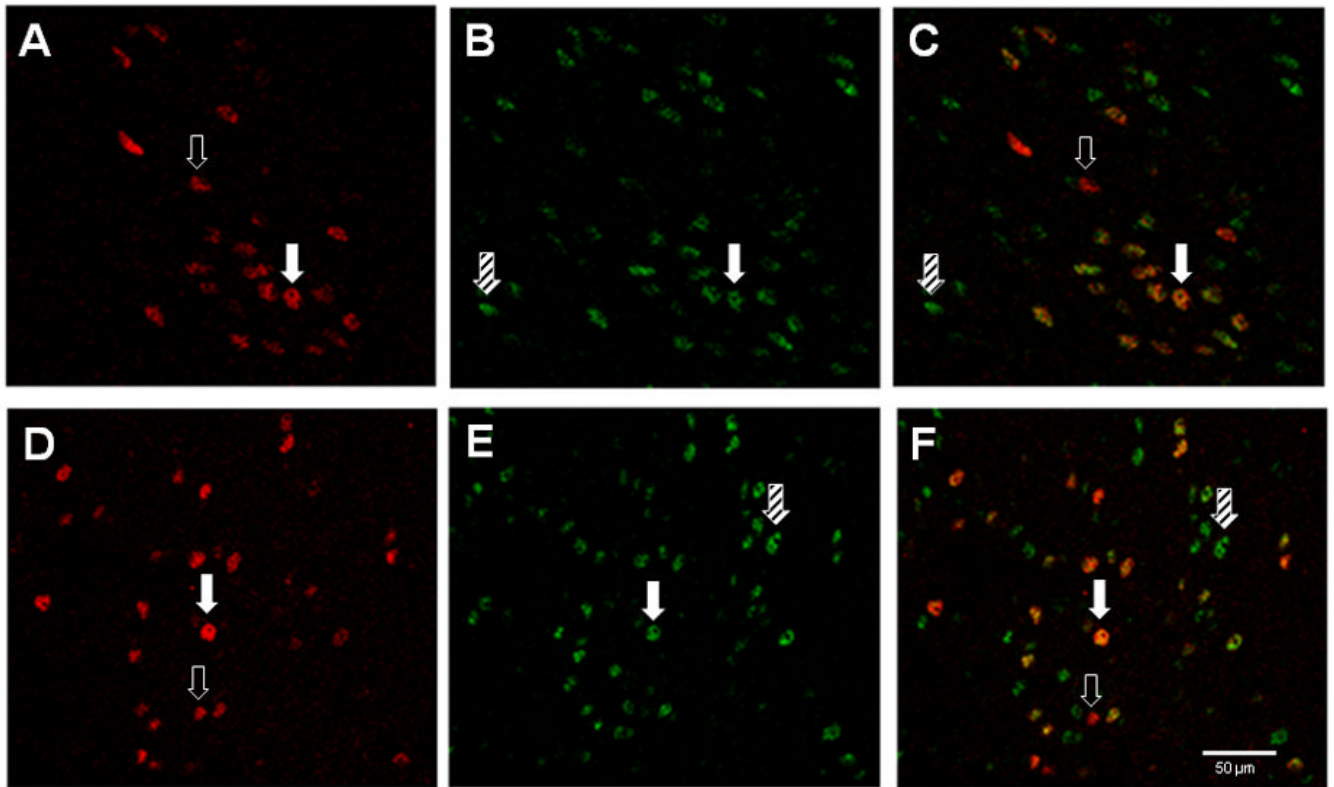


Figure 2.

Coexpression of SRC-2 and estrogen receptors (ER) in cells in the ventromedial nucleus of the hypothalamus (A–C) and arcuate nucleus (D–F). Sections were simultaneously immunostained for ER α (A and D) and SRC-2 (B and E). Overlaid images from the VMN (C) and ARC (F) show cells expressing both ER α and SRC-2. Open arrows point to cells containing ER α only (red), hatched arrows point to cells containing SRC-2 only (green) and solid arrows point to cells expressing both ER α and SRC-2 (orange/yellow). Magnification bar = 50 μ m

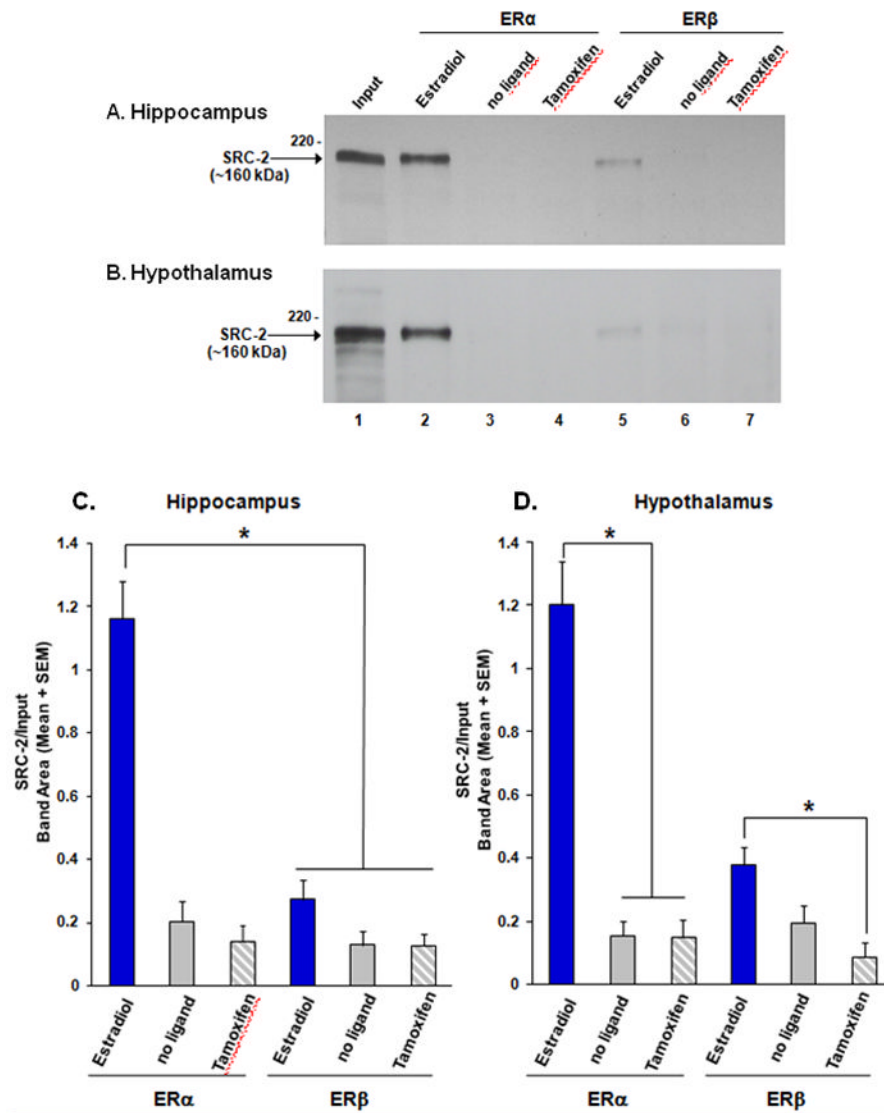


Figure 3.

SRC-2 from rat hypothalamic whole cell extracts associates with ER α , but not ER β , in a ligand-dependent manner. SRC-2 from A) hippocampus or B) hypothalamus interacts with ER α in the presence of estradiol (lane 2), but not in the absence of ligand (lane 3) or in the presence of the SERM, tamoxifen (lane 4). SRC-2 interacts weakly with ER β bound to estradiol (lane 5) with little to no interaction in the absence of ligand or tamoxifen (lanes 6 & 7). Inputs (2% of total) of SRC-2 from hippocampal or hypothalamic extracts are shown in lane 1.

C) SRC-2 from hippocampus physically associates with ER α bound to estradiol, but not in the absence of ligand or in the presence of tamoxifen. In contrast, SRC-2 interacts weakly with ER β . D) Hypothalamic SRC-2 interacts with ER α , and to a much lesser extent with ER β , in a ligand dependent manner. * $p < 0.05$, $n = 4-5$ per treatment group.

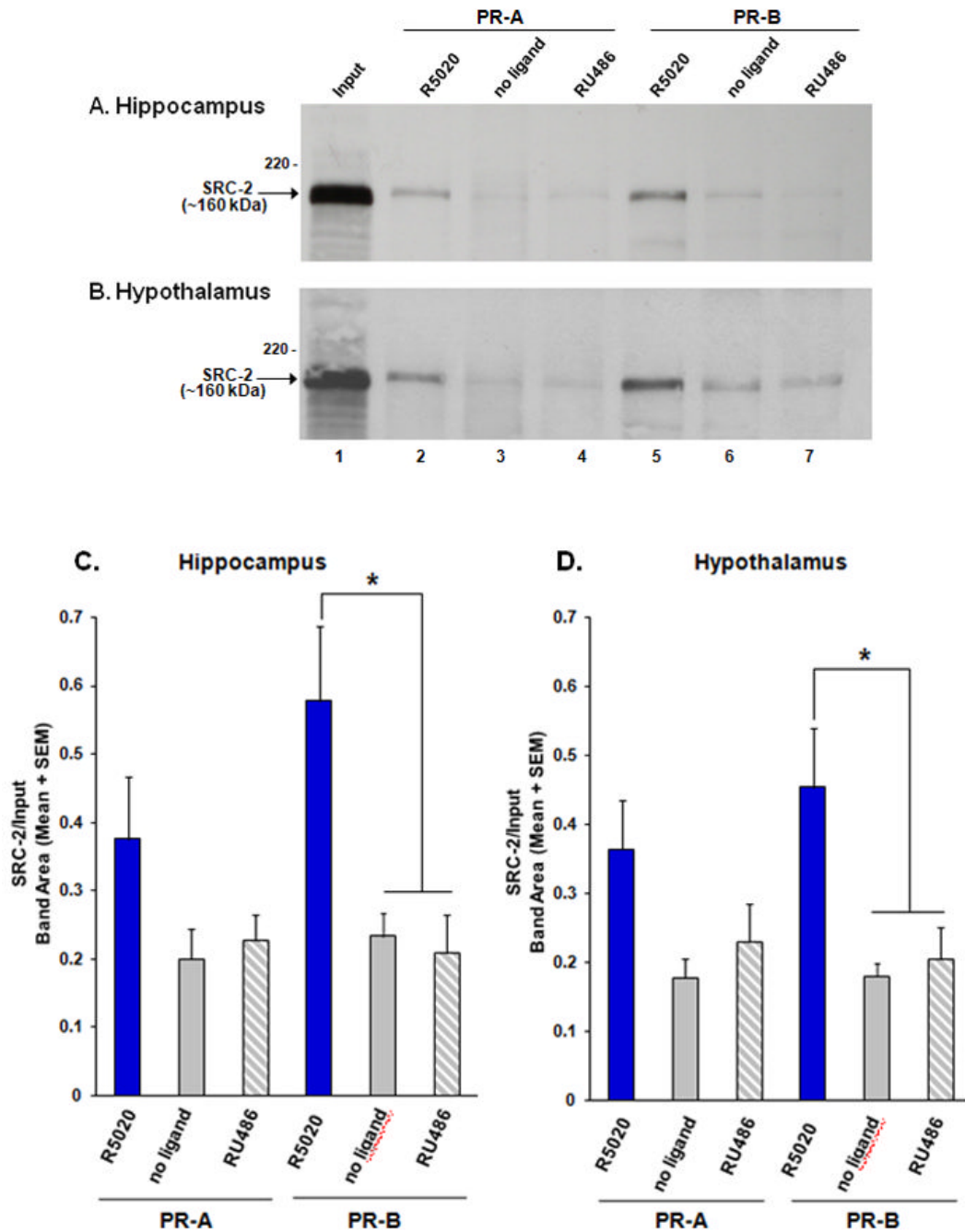


Figure 4.

Progestin receptor (PR) interactions with SRC-2 from brain. SRC-2 from A) hippocampal or B) hypothalamic whole cell extracts interacts with PR-B, and to a lesser extent PR-A, in the presence of the agonist R5020, but not in the absence of ligand or in the presence of the SPRM, RU486. Inputs (2% of total) of SRC-2 from hippocampal or hypothalamic extracts are shown in lane 1.

SRC-2 from the C) hippocampus and D) hypothalamus interacts with PR-B in the presence of the agonist R5020, but not in the absence of ligand or in the presence of the SPRM, RU486. SRC-2 from each brain region interacts with PR-A to a lesser extent. * $p < 0.05$, $n = 8$ per treatment group.

Table 1Mass Spectrometry Identification of SRC-2 Bound to Estradiol-Treated ER α

Scan#	Peptide Sequence	MH+z	chargeXcorr	Δ Cn	Sp	RSp	Ions
2740	SDVSTGQGVIDK	1292.63285	2	3.47	0.40	1624.0	1 19/24
3248	ELNQESSGTAPGSEVTVK	1832.88723	2	3.97	0.60	530.3	1 18/34
4126	LLQDSSSPVDLAK	1372.73184	2	3.40	0.40	1830.2	1 19/24