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Nuclear Receptor Coactivators Modulate Hormone-Dependent Gene Expression in Brain and Female Reproductive Behavior in Rats

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Gonadal steroid hormones act in the brain to elicit changes in gene expression that result in profound effects on behavior and physiology. A variety of *in vitro* studies indicate that nuclear receptor coactivators are required for efficient transcriptional activity of steroid receptors. Two nuclear receptor coactivators, steroid receptor coactivator-1 (SRC-1) and cAMP response element binding protein-binding protein (CBP), have been shown to act in concert to enhance ER activity *in vitro*. In the present study, we investigated the function of these important nuclear receptor coactivators in estrogen action in rodent brain. Reduction of SRC-1 and CBP protein in brain disrupted ER-mediated activation of the behaviorally relevant progestin receptor gene. Furthermore, we found that SRC-1 and CBP function in brain to modulate the expression of hormone-dependent female sexual behavior. These findings indicate that these nuclear receptor coactivators function in brain to modulate ER transcriptional activity and the expression of hormone-dependent behavior. (*Endocrinology* 143: 436–444, 2002)

STEROID HORMONES ARE essential for growth, development, and reproduction. Many of the biological effects of steroid hormones are mediated by their respective intracellular steroid receptors, which are members of a nuclear receptor superfamily of transcriptional activators (1, 2). Disruption of hormone action during development or in adulthood can result in devastating endocrine disorders that have profound effects on reproductive physiology (3). While many of these endocrine disorders are caused by mutations in receptors or altered hormone levels, recently, some of these disorders have been attributed to defects in nuclear receptor coactivators (4–6), which are critical for nuclear receptor activity. Thus, it is essential to understand the function of these nuclear receptor coactivators in reproductive behavior and physiology.

The ovarian hormones, E2 and progesterone, act in the brain to regulate rodent female reproductive behavior and physiology (7–10). E2 and progesterone elicit many of their effects on female reproduction by binding to their respective receptors, ER and PR, in specific regions of the brain. Upon binding hormone, steroid receptors undergo a conformational change that causes dissociation from an inactive oligomeric complex composed of heat shock proteins and immunophilins, transforming the receptor to the active form (11). The activated receptors then dimerize (12) and bind to the steroid response element of steroid-target genes to alter the rate of gene transcription (13). A classic example of a steroid-responsive gene is the induction of the PR gene by E2 in a variety of hormone-responsive tissues, including brain (14–17). This ER-dependent induction of PR gene expression is thought to occur *via* an estrogen response element in the promoter of the PR gene (18–20). In the brain, E2 induces PR expression in the medial preoptic area (mPOA) and ventromedial nucleus of the hypothalamus (VMN) (16, 17, 21, 22), sites known to regulate hormone-dependent reproductive behavior (8, 21, 23–27). Moreover, E2 induction of PR in the VMN is required for progesterone-facilitated reproductive behavior (21).

Nuclear receptor coactivators have been shown to dramatically enhance the transcriptional activity of nuclear receptors in a variety of *in vitro* studies (28–31). As proposed by Liu *et al.* (32), nuclear receptor coactivators are thought to enhance steroid-dependent gene transcription *via* a two-step mechanism in which coactivators bridge the steroid receptor complex with the basal transcription machinery and remodel chromatin structure through their intrinsic histone acetyltransferase activity (33, 34). Steroid receptor coactivator-1 (SRC-1, also known as NCoA-1), which was the first coactivator of steroid receptors to be discovered, belongs to a larger p160 family of nuclear receptor coactivators (29). This family of coactivators also includes SRC-2 (NCoA-2/GRIP-1/TIF-2) (35, 36) and SRC-3 (RAC3/AIB1/pCIP/ACTR/TRAM1) (5, 37, 38). *In vitro*, SRC-1 enhances the transcriptional activity of a variety of nuclear receptors, including ERα and β and PR, in a ligand-dependent manner (29, 39). SRC-1 is expressed in a variety of hormone-responsive tissues including brain, uterus, prostate, and breast (40–47). SRC-1

Abbreviations: Arc, Arcuate nucleus; CBP, CREB binding protein; CREB, cAMP response element binding protein; mPOA, medial preoptic area; ODN, oligodeoxynucleotide; SRC-1, steroid receptor coactivator-1, also known as NCoA-1; TBS, Tris-buffered saline; TBS-T, TBS containing 0.05% Tween-20; VMN, ventromedial nucleus of the hypothalamus.

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null mutant mice, while fertile, exhibit decreased growth of steroid-responsive tissues, such as the uterus, prostate and testes, compared with wild-type mice (42). Interestingly, SRC-2 is up-regulated in these SRC-1 knockouts, suggesting that SRC-2 may partially compensate for the loss of SRC-1 in these mice (42). SRC-3 null mice have disrupted female reproductive physiology, including delayed puberty and prolonged estrous cycles compared with their wild-type littermates (48).

cAMP response element binding protein (CREB) binding protein (CBP) was initially discovered to be a transcriptional activator of CREB (49, 50). More recently, CBP has been found to function as an integrator of nuclear receptors with other cell signaling pathways (50, 51). Studies reveal that CBP and SRC-1 function together to increase ER and PR function and transcriptional activity *in vitro* (52, 53). It has been suggested that SRC-1, as well as other members of the p160 family, provides a platform to recruit CBP to the coactivator complex (31, 43).

A few recent studies have begun to investigate the role of coactivators in hormone action in brain. SRC-1 mRNA is expressed throughout the brain, including high levels in the hypothalamus, hippocampus, cerebellum, thalamus, and amygdala (40, 46, 47, 54). In addition, two isoforms of SRC-1, SRC-1a and the truncated SRC-1e, are expressed differentially in the rat brain (46). SRC-1a is found in high levels in the hypothalamus, whereas SRC-1e predominates in areas such as the nucleus accumbens, thalamus, and amygdala (46). Moreover, SRC-1 has recently been shown to be critical for hormone-dependent development of the mammalian brain (45). Reduction of SRC-1 expression in the sexually dimorphic nucleus of the preoptic area, by antisense oligonucleotides to SRC-1 mRNA, disrupts the estrogen-dependent sexual differentiation of this nucleus (45). Whereas SRC-2 mRNA has been observed in cerebellum (55) and whole brain (42), one *in situ* hybridization study has failed to detect SRC-2 in brain (46). SRC-3 appears to be expressed in hippocampus, cerebellum, and olfactory bulb (48). Finally, immunocytochemical studies reveal that CBP protein is widely expressed in brain, and is found in high levels in the hypothalamus, preoptic area, thalamus, amygdala, hippocampus, cortex, and cerebellum (56). While homozygous knockouts for CBP die *in utero*, heterozygote adults have memory deficits, suggesting CBP is required for normal brain development (57).

In order for nuclear receptor coactivators to enhance steroid receptor transcriptional activity in brain, both coactivator and receptor must be present in the same cell. Indeed, recent studies in our lab have found that PR- and ER-containing neurons coexpress SRC-1 and CBP in the VMN and mPOA (58), sites known to regulate hormone-dependent reproductive behavior (7, 8, 21, 23–27). In support of these findings, SRC-1 and ER are coexpressed in rat mammary tissue (41).

While much is known about the molecular mechanisms of coactivators in hormone action *in vitro*, we are only beginning to understand the function of these important cofactors in hormone-dependent gene expression in brain and the regulation of behavior (59). The present experiments tested the hypotheses that SRC-1 and CBP are involved in ER-mediated transactivation of the progestin receptor gene in brain. Furthermore, we asked if these coactivators function in specific cell populations in the brain to modulate hormone-dependent female sexual behavior. In this report, we demonstrate that decreasing expression of SRC-1 and CBP in the VMN disrupts hormone-dependent PR gene expression in brain and female sexual behavior.

Materials and Methods

Experimental animals

Adult female (175–200 g) and male (300–350 g) Sprague Dawley rats from Charles River Breeding Laboratories, Inc. (Wilmington, MA) were housed singly in a 14-h light, 10-h dark cycle, with lights off at 1000 h. Animals were given food and water *ad libitum*. Female rats were anesthetized with a combination of chloral hydrate (170 mg/kg) and pentobarbital (35 mg/kg), ovariectomized, and then placed in a stereotaxic apparatus for implantation of 28-gauge bilateral cannulae aimed just dorsal to the VMN [anterior-posterior, −3.14 mm; medial-lateral, 1.0 mm; dorsal-ventral, −9.0 mm from Paxinos and Watson (60)]. This cannulae placement allowed infusion of oligodeoxynucleotides into the VMN without destroying VMN cell bodies or fibers. Only animals in which infusion cannulae descended to a depth within 1 mm of the fornix, and medial to the fornix, were included in immunocytochemical and behavioral analyses. A 1-wk recovery period followed to allow clearing of endogenous hormones. During this week, the animals were handled and dummy cannulae were removed and replaced to prevent clogging of guide cannulae. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts (Amherst, MA).

Exp 1

Antisense oligodeoxynucleotide treatment. Antisense oligodeoxynucleotides (Oligos Etc., Wilsonville, OR) (21-oligomer) specific to SRC-1 (5′ CTG-TCC-CCA-AGG-CCA-CTC-ATG 3′, targeting both SRC-1a and SRC-1e isoforms) and CBP (5′ CAG-CAA-GTT-CTC-GGC-CAT-CTT 3′) mRNA were designed to span the translation start site to prevent translation of these coactivators. Control oligodeoxynucleotides (Oligos, Etc.) contained the same bases in scrambled sequence, and were confirmed to not cross-hybridize with other rodent gene sequences by a Basic Local Alignment Search Tool search of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The chimeric oligodeoxynucleotides (ODNs) used in these experiments contain limited phosphorothioate linkages resulting in increased resistance to nuclease degradation of the ODNs and increased RNase H activity, which allows for more efficient digestion of target RNA strands. Finally, phosphorothioate-linked antisense oligonucleotides have been reported to be stable in rodent brain for up to 24 h (61).

One week following ovariectomy, each animal was administered one ODN infusion at 0900 h on 3 d consecutively (d 1, 2, and 3). Each rat was infused with both SRC-1 (1 μg) and CBP (1 μg) antisense ODN in 2 μl of saline to one side of the VMN and 1.0 μg of each of the scrambled control ODNs to the contralateral VMN (n = 14). To determine the effect of each coactivator alone, another group of animals was infused with either antisense ODNs to SRC-1 (1 μg, n = 7) or CBP (1 μg, n = 7) mRNA and the corresponding scrambled control ODN (1 μg) infused into the contralateral VMN. Bilateral infusions were administered simultaneously and cannulae left in place for 1 min after infusion to prevent ODNs from being drawn up into the guide cannulae. To induce PR expression, animals were injected with 17-β E2 benzoate (EB; 2 μg dissolved in 0.1 ml of sesame oil, sc) on d 2 (15). Forty-eight hours following E2 injection (d 4), animals were injected with an overdose of an anesthetic mixture (ip, 425 mg/kg chloral hydrate and 89 mg/kg pentobarbital) and perfused intracardially with saline for one minute, followed by 2% acrolein in 0.1 M phosphate buffer for 14 min at a fixative flow rate of 25 ml/min. Brains were removed and stored in 20% sucrose solution for 48 h at 4 C and transverse sections were cut from the POA through the hypothalamus at 40 μm on a freezing microtome. Brain sections were stored in cryoprotectant at −20 C until immunocytochemistry was performed.

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Progestin receptor immunocytochemistry

Sections were initially rinsed in 0.05 M Tris-buffered saline (TBS), followed by a pretreatment of 1% sodium borohydride for 10 min to remove residual aldehydes. Tissue was then rinsed in TBS and incubated in a solution of 1% H_2O_2, 20% normal goat serum, and 1% BSA in TBS for 20 min to decrease nonspecific staining and reduce endogenous peroxidase activity. Sections were incubated for 48 h in 1:1000 dilution of a rabbit polyclonal primary antibody generated against the DNA binding domain of human PR (A0098, DAKO Corp., Carpinteria, CA) in TBS containing 0.02% sodium azide (NaN_3), 1% normal goat serum, 0.1% gelatin, and Triton-X (pH 7.6 at 4 C). Tissue was rinsed in TBS containing NaN_3, gelatin, and Triton-X before incubation in a biotinylated goat antirabbit IgG (3 μg/ml, Vector Laboratories, Inc., Burlingame, CA) in TBS containing NaN_3 and Triton X-100 and 1.5% normal goat serum for 90 min. Tissue was rinsed in TBS containing NaN_3, gelatin, and Triton X-100 followed by rinsing in TBS. Sections were then incubated for 90 min in TBS containing 1% avidin DH:biotinylated horseradish peroxidase H complex (Vectastain ABC Elite Kit, Vector) followed by rinsing in TBS. Finally, sections were exposed to 0.5% diaminobenzine with 3% hydrogen peroxide with TBS for approximately 3 min. The sections were rinsed in TBS and then mounted on microscope slides and coverslipped using DePeX mounting medium (Electron Microscopy Sciences, Fort Washington, PA).

PR-immunoreactive cells in the VMN, arcuate nucleus, and mPOA [Plates 32, 32, and 20, respectively, as defined by Paxinos and Watson (60)] were analyzed under 200× magnification using a Leitz Dialux 20 microscope (Leitz, Wetzler, Germany) with an MTI CCD72 camera (Dage MTI, Michigan City, MI) connected to a Macintosh G3 computer. The number of immunoreactive cells was quantified using the NIH Image 1.62 program as described previously (62). Briefly, the microscope was placed in Kohler illumination and the gain and black levels of the camera were adjusted so that the pixel density of a blank section of the slide measured 2–10 U and black measured 252 U. The threshold for detection of specific immunoreactivity was determined as a function of background. Cells were considered immunopositive at a density four times that of the background threshold and if they were greater than 10 pixels but smaller than 200 pixels in total area.

Analysis of nuclear receptor coactivator protein expression

To determine if antisense treatment decreased SRC-1 and CBP protein expression in the VMN, immunocytochemistry for nuclear receptor coactivators was done using the procedure described above with the following modifications. For immunocytochemical detection of SRC-1, 1135-H4, a mouse monoclonal primary antibody generated against amino acids 477–947 of human SRC-1 (kindly provided by Dean Edwards, University of Colorado Health Sciences Center; and Bert O'Malley, Ming Tsai, and Sergio Oñate, Baylor College of Medicine), and biotinylated goat antimouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) were used. For analysis of CBP expression, a rabbit polyclonal antibody generated against amino acids 162–176 of human CBP (PA1–847, Affinity BioReagents, Inc., Golden, CO) was used. SRC-1- and CBP-immunoreactive cells in the VMN were analyzed as described above.

To determine if antisense decreased CBP protein expression as detected by Western blot, adult females were bilaterally infused into the VMN with antisense to both CBP and SRC-1 mRNA (n = 3) or scrambled control (n = 6) ODNs. Using a cryostat, frozen brains were sectioned at 300 μm onto glass slides. The ventromedial hypothalamic area was dissected out on a chilled surface and stored frozen until homogenization with ice-cold lysis buffer consisting of 50 mM Tris-HCl, 1% Na-deoxycholate, 0.25% NP-40, 150 mM NaCl, 1 mM EDTA, and protease inhibitors (1 μg/ml of aprotinin, leupeptin, and pepstatin; 1 mM pheynylmethylsulfonyl fluoride). Following tissue homogenization, samples were centrifuged at 2,000 × *g* for 30 min at 4 C. The supernatant fraction was collected, and protein concentration determined by Bradford assay. Briefly, protein concentration was quantified by comparing colorimetric intensity of the reaction product from each sample (performed in triplicate) with a set of known protein standards. Twenty micrograms of total protein from each rat were electrophoresed on a pre-cast SDS-polyacrylamide gel (8–16% Tris glycine) and transferred to a polyvinylidenedifluoride membrane. Membranes were washed briefly in 0.1 M TBS and blocked for 1 h in 0.1 M TBS containing 5% nonfat dry milk with constant agitation at room temperature. Membranes were then incubated with a rabbit polyclonal antibody generated against human CBP peptide (PA1–847) at a dilution of 1:3000 in TBS containing 0.05% Tween-20 (TBS-T) for 3 h at room temperature. Following washes in TBS-T, membranes were incubated in a goat antirabbit HRP-linked secondary antibody (1:2000, Cell Signaling Technology, Beverly, MA) for 30 min at room temperature, and then washed in TBS-T. Immunoreactive bands were detected using an enhanced chemiluminescence kit (ECL kit; New England Biolabs, Inc., Beverly, MA) and membranes exposed to film (Hyperfilm-ECL, Amersham Pharmacia Biotech, Arlington Heights, IL). Films were placed on a light box, and a digital image was captured. The image was analyzed using the public domain NIH Image program. Within this program, a gel plotting macro was used to perform densitometric analysis of CBP, which integrates both the optical density and the area of immunolabeling of each band. The numbers represent the number of pixels in the area under the curve.

Cell death analysis

To determine whether antisense infusions damaged brain tissue in the VMN, nissl-stained matched VMN sections were analyzed for cell death. Pyknotic cells were counted at 1,000× magnification using a Nikon Optiphot-2 microscope. Pyknotic cells were determined by their condensed size and intensified staining relative to surrounding neurons or glial cells (63). Photomicrograph of healthy and pyknotic cells were taken under 1,000× magnification with a Nikon FX-35DX 35 mm camera with Kodak 160 Tungsten color slide film (Eastman Kodak Co., Rochester, NY).

Exp 2

Female sexual behavior. Rats were ovariectomized and cannulated as described in Exp 1. After a 1-wk recovery period, females were primed with 2 μg of EB and 44 h later with 300 μg of progesterone. Four hours later, females were tested for sexual receptivity with an intact male. Only females that were sexually receptive, as indicated by displaying lordosis, remained in the study. One week later, females were administered bilateral infusions of ODNs into the VMN at 900 h on d 1, 2, and 3. One group of females received bilateral infusions of antisense ODNs to SRC-1 and CBP mRNA (1 μg of each, n = 12), while a second group received bilateral infusions of scrambled control ODNs (1 μg of each control ODN, n = 9). On d 2, animals were injected with 2 μg of EB. On d 4 animals were injected with 300 μg of progesterone and tested 4 h later for sexual behavior.

Females were tested for lordosis by being placed in a Plexiglas arena (53 cm diameter) with an intact sexually experienced male. Testing was performed under a dim red light during the dark phase of the light:dark cycle, with each female receiving 10 mounts by the male. For each mount, the lordosis response and rejection behaviors (kicking, biting, or fighting) were recorded by two experimenters who were blind with regard to treatment group. Each lordosis response was measured on a scale from 0 (no dorsoflexion) to 3 (maximal dorsoflexion) (27, 64). For each female, lordosis intensity (LI, total points scored/number of mounts) and the lordosis quotient [(LQ, number of lordosis responses/number of mounts) × 100] were calculated. All behavior sessions were video taped.

Statistical analysis

Cell counts of each brain area were analyzed using a two-tailed paired *t* test with the statistical software program, SigmaStat (SPSS, Inc., San Rafael, CA). For clarity in representing the decrease in cell numbers in the within animal design, data are expressed as the ratio of PR cells in antisense-treated VMN to PR cells in scrambled control-treated VMN in Table 1. Analyses of Western blot data and behavioral scores were done using two-tailed *t* tests. Differences were considered significant at a probability of less than 0.05.

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Results

Exp 1: nuclear receptor coactivators modulate E2-induced PR expression

Infusion of antisense ODNs to both SRC-1 and CBP mRNA into one side of the VMN decreased the number of E2-induced PR-immunoreactive cells in this nucleus compared with the control-treated contralateral VMN (Fig. 1 and Table 1). The effect of antisense ODNs on E2-induced PR was observed consistently in the VMN of all animals, with many of the animals showing a decrease in the ventrolateral aspect of the VMN (Fig. 1), a subregion thought to be important in the regulation of progesterone-facilitated lordosis (25). In contrast, when antisense ODNs to only SRC-1 mRNA, or only CBP mRNA, were infused into the VMN, no differences in PR expression were observed between scrambled control- and antisense-treated nuclei (Table 1). Taken together, these data suggest that both SRC-1 and CBP are important in modulating ER-mediated activation of PR gene expression. No differences in the number of PR-immunoreactive cells in the VMN were observed when treated with scrambled control ODNs (260.5 ± 59.5) or H_2O (255.5 ± 61.5), suggesting that scrambled control ODNs had no nonspecific effect on PR gene expression. When cannulae were placed outside the VMN and did not meet our acceptable criteria as described in *Materials and Methods*, no differences in PR expression in the VMN were found between antisense- (81 ± 32.6 PR-IR cells) and scrambled control- (103 ± 47.6, $P = 0.28$) treated animals. Within the animals with acceptable cannulae placements, there was no correlation between cannulae placement and degree of E2 induction of PR or reproductive behavior.

No difference in the number of E2-induced PR-immunoreactive cells was detected between matched sides of the arcuate nucleus of the hypothalamus (ARC) when antisense ODNs to both SRC-1 and CBP (148.3 ± 21.5) or scrambled control ODNs (147.6 ± 20.3) were infused into the VMN (n = 14), indicating that the ODNs did not diffuse into the ARC and alter E2-induced PR gene expression in this nucleus. Likewise, analysis of the mPOA revealed no differences in the number of PR-immunoreactive cells between antisense-treated (141.6 ± 32.2) and scrambled control-treated (134.4 ± 27.6) nuclei (n = 14).

One possibility for coactivator antisense decreasing E2-induced PR expression is that the antisense ODNs may cause nonspecific cell death in the VMN. However, we found no differences in the number of pyknotic cells (63) in the antisense treated VMN (4.3 ± 0.8, n = 6) compared with the scrambled control treated VMN (3.8 ± 1.0, n = 4) (Fig. 2), indicating that a reduction in PR protein expression was not due to increased cell death in the this brain area. These findings are consistent with other studies that found no increase in cell death in brain due to antisense ODN treatment (65). Furthermore, in the present study tissue damage did not appear to extend into the VMN, as infusion cannulae were placed just dorsal to this nucleus (Fig. 3).

Immunostaining for SRC-1 was performed to confirm that antisense treatment reduced SRC-1 protein expression. Infusion of the VMN with antisense ODNs to both SRC-1 and CBP mRNA decreased the number of SRC-1 protein-expressing cells relative to the contralateral control-treated VMN (Fig. 4A), confirming that antisense treatment was effective in decreasing the target protein. While a difference in the number of CBP-immunoreactive cells in the VMN was not detected by immunocytochemistry (antisense-treated VMN = 104.0 ± 39.4 CBP-IR cells *vs.* control-treated VMN = 99.4 ± 50.7, n = 5), we did observe a difference in CBP protein expression in the ventromedial hypothalamus as detected by Western blot analysis. As shown in Fig. 4B, CBP protein levels were lower in antisense-treated animals compared with controls. These data suggest that our antisense technique was indeed effective in decreasing CBP protein expression in the ventromedial hypothalamus, an area that contains the VMN. The inability to detect a decrease in CBP by immunocytochemistry may be due to the difficulty in optimizing this technique for a protein that is widely expressed in brain (56). In further support of antisense disrupting SRC-1 and CBP expression in the female brain, the present effects of coactivator antisense on gene expression

TABLE 1. Antisense to both SRC-1 and CBP mRNA reduces E2-induced PR gene expression in the VMN

ODNs to SRC-1 and CBP	ODNs to SRC-1	ODNs to CBP
0.82 ± 0.06[a]	1.0 ± 0.10	0.93 ± 0.12

Data are presented as ratio of the number of PR-immunoreactive cells in the antisense-treated VMN relative to the control-treated contralateral VMN. Infusion of antisense ODNs to both SRC-1 and CBP mRNA decreased E2-induced PR gene expression in the antisense-treated side of the VMN relative to the control-treated contralateral side (n = 14). No differences in PR expression were observed when antisense ODNs to SRC-1 mRNA only (n = 7), or CBP mRNA only (n = 7), were infused into the VMN compared with the control-treated contralateral side.

[a] $P < 0.05$; two-tailed paired *t* test.

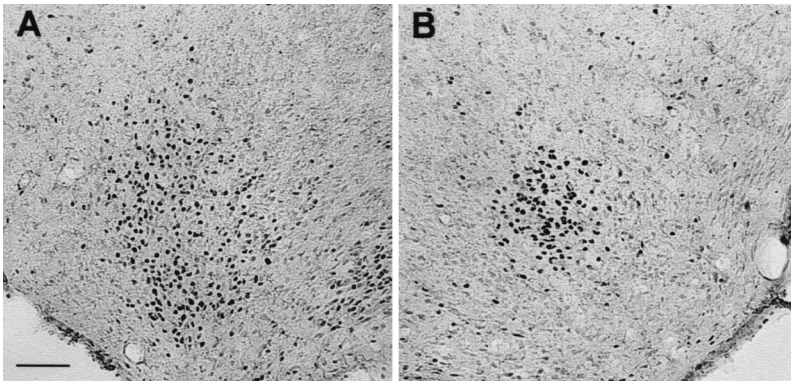

FIG. 1. Antisense ODNs to coactivator mRNA decreases E2-induced PR gene expression in the VMN. The VMN of a female rat treated (A) on one side with scrambled control ODNs and (B) antisense ODNs to SRC-1 and CBP mRNA on the contralateral side. *Scale bar*, 50 μm.

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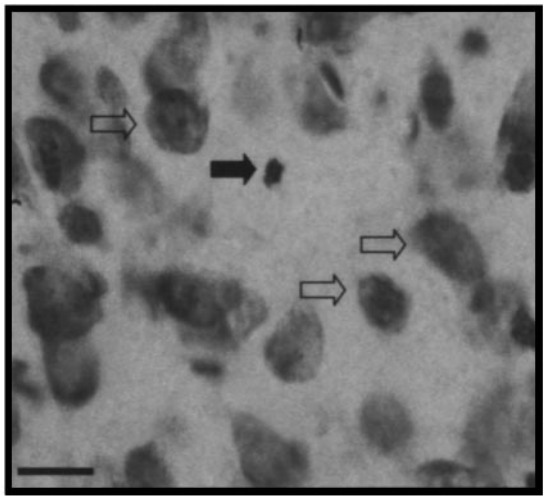

FIG. 2. Photomicrograph of healthy and pyknotic cells in the VMN. A Nissl-stained section with several healthy cells (*open arrows*) and one pyknotic cell (*closed arrow*) in the VMN of a scrambled ODN-treated female. No differences were found in the number of pyknotic cells between antisense- or scrambled control-treated VMN (see *Results* section). *Scale bar*, 10 μm.

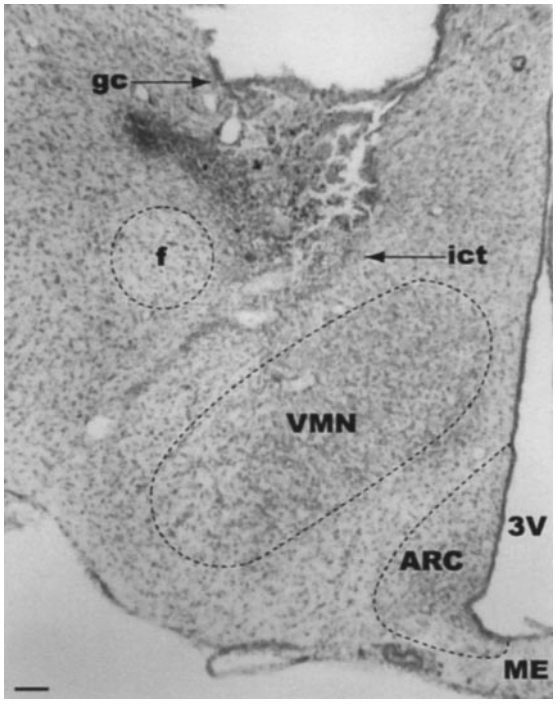

FIG. 3. Histology of infusion cannulae placement dorsal to the VMN. Nissl-stained section shows that guide cannulae (gc) were implanted so that infusion cannula tips (ict) were just dorsal to the VMN. f, Fornix; ARC, arcuate nucleus; 3V, third ventricle; ME , median eminence. *Scale bar*, 100 μm.

were observed only when antisense to both SRC-1 and CBP were used.

Exp 2: nuclear receptor coactivators influence hormone-dependent reproductive behavior

To test the hypothesis that nuclear receptor coactivators function in the brain to modulate hormone-dependent female reproductive behavior, we used our antisense technique to decrease coactivator expression in the behaviorally-relevant VMN. Animals that received infusions of antisense ODNs to both SRC-1 and CBP mRNA in the VMN had a decrease in lordosis intensity compared with animals treated with scrambled control ODNs (Table 2). As assessed by the number of chamber midline crossings, there were no differences in general locomotor activity between scrambled control (15.8 ± 1.2)- and antisense (19.9 ± 2.2)-treated rats, suggesting that a decrease in lordosis intensity by antisense treatment was not due to differences in locomotor activity. These data extend our findings from Exp 1 and provide further evidence that SRC-1 and CBP influence hormone action in the VMN. Moreover, these results suggest these nuclear receptor coactivators function in the VMN to mod-

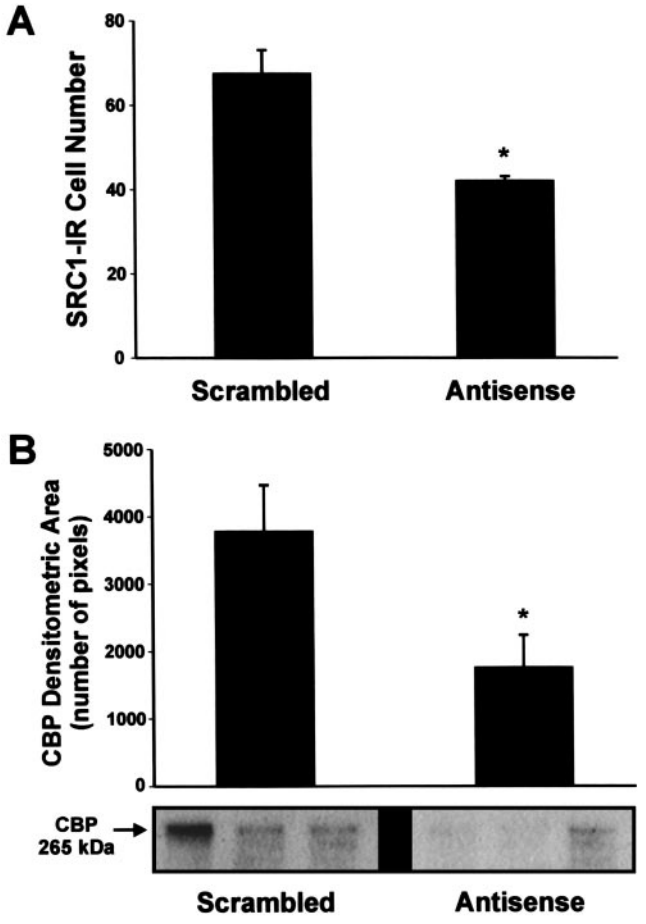

FIG. 4. Antisense oligodeoxynucleotides to SRC-1 and CBP mRNA decrease nuclear receptor coactivator protein expression in the ventromedial hypothalamus. A, Histogram represents immunocytochemical analysis of the number of SRC-1-immunoreactive (SRC1-IR) cells in the corresponding VMN of females treated on one side with scrambled control ODNs and antisense ODNs to both SRC-1 and CBP mRNA on the contralateral side (n = 4). *, $P < 0.02$, paired t test. B, Histogram represents Western blot analysis of the densitometric area of CBP-immunoreactive bands in the ventromedial hypothalamus of females treated bilaterally with scrambled control ODNs (n = 7) and females treated bilaterally with antisense ODNs to both SRC-1 and CBP mRNA (n = 6). *Inset* is a photomicrograph of three representative animals from each group showing a reduction of CBP protein expression by antisense ODNs. *, $P < 0.04$, t test.

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TABLE 2. SRC-1 and CBP function in the VMN to modulate the expression of hormone-dependent female reproductive behavior

	Lordosis Intensity (mean ± SEM)	Lordosis Quotient % (mean ± SEM)
Scrambled Control ODNs	2.3 ± 0.07	88.9 ± 4.0
SRC-1 and CBP Antisense ODNs	1.9 ± 0.10[a]	86.7 ± 4.0

Animals treated with bilateral infusions of antisense ODNs to both SRC-1 and CBP mRNA (n = 12) in the VMN had a decrease in lordosis intensity compared with scrambled control ODN-treated animals (n = 9).

[a] $P < 0.01$; two-tailed t test.

ulate the expression of hormone-dependent female reproductive behavior.

We detected no effect of antisense treatment on the lordosis quotient (Table 2). Our data suggest that while the intensity of lordosis was altered by coactivator antisense treatment, the frequency of lordosis was not changed. No difference was detected in the rejection quotient in antisense-treated (0.15 ± 0.04) and control-treated (0.22 ± 0.07) rats.

Discussion

The nuclear receptor coactivators, SRC-1 and CBP, have been shown previously to act in concert to enhance ER and PR transcriptional activity *in vitro* (52). In the present studies, we used antisense ODNs to decrease SRC-1 and CBP protein expression in brain to test the hypothesis that these coactivators function in hormone action in an *in vivo* system. Indeed, we found that SRC-1 and CBP are important in mediating steroid-dependent transcriptional activity in brain and the expression of hormone-dependent reproductive behavior. Infusion of antisense ODNs to SRC-1 and CBP mRNA into the VMN decreased E2-induced PR gene expression in this behaviorally-relevant brain region (Fig. 1 and Table 1). These findings indicate a functional role for coactivators in hormone-dependent gene expression in the brain. No effect on PR gene expression was observed following infusion of antisense to either SRC-1 or CBP mRNA alone. The present finding that antisense to SRC-1 mRNA alone was not effective in altering hormone-dependent gene expression in brain is consistent with the fact that SRC-1 knockout mice remain fertile (42). While it is possible that our assay was not sensitive enough to detect an effect of antisense to only one coactivator, it may be that suppression of both nuclear receptor coactivators is required to alter ER action. Thus, our data extend previous *in vitro* findings (52) by providing evidence that SRC-1 and CBP function together to modulate ER activity in brain.

One limitation of the present experiments is that they do not exclude the possibility that coactivator antisense ODNs are affecting E2-induced PR cells indirectly by acting on cells that lack ER. However, virtually all hypothalamic E2-induced PR cells contain ERα (66). Furthermore, neuroanatomical data from our lab reveal that the majority of E2-induced PR cells in the VMN coexpress SRC-1 and/or CBP (58). Taken together, the present findings are consistent with the concept of coactivator antisense ODNs acting directly on ER-containing cells in the VMN. Based on these findings, and a variety of *in vitro* studies, we propose that nuclear receptor coactivators modulate ER-mediated induction of PR gene expression in brain. Activated ER dimers bind to an estrogen response element on the PR gene (18, 19). SRC-1 and CBP, as well as other coactivators, bridge the ER dimer with the basal transcription machinery (28, 30, 31, 33, 34), and facilitate efficient transcription of the PR gene. While it is possible that the present CBP effects are via a steroid receptor independent pathway, our finding that CBP antisense decreases E2-induced PR expression only in conjunction with SRC-1 antisense, and not alone, suggests that CBP is acting as a coactivator of ER. In support, *in vitro* studies indicate that CBP coactivates ERα and β (51, 52, 67) and interacts with SRC-1 (51, 68), suggesting a model in which ER forms a ternary complex with SRC-1 and CBP at target gene promoters (29, 51, 52). Finally, the activity of other transcription factors in which CBP serves as a coactivator, such as CREB, are not influenced by SRC-1 (29).

While in the present studies antisense ODNs to SRC-1 and CBP mRNA decreased E2-induced PR gene expression in the VMN, we did not expect an elimination of PR induction for the following reasons. While many PR-containing cells in the VMN express SRC-1 and/or CBP (58), it is important to note that not all PR cells in this nucleus contain both SRC-1 and CBP. Therefore, one would not expect antisense to these coactivators to influence all E2-induced PR cells in the VMN. In addition, it is likely that other coactivators function in modulating ER action in brain. For example, recent studies of SRC-3 knockout mice have implicated this coactivator in female reproductive physiology (48). Furthermore, other coactivators, such as TR-associated protein, receptor interacting protein 140, and vitamin D receptor interacting protein, can also enhance transcriptional activity of ER α and β *in vitro* (69–75). These coactivators may also function in ER action in brain and could be up-regulated to compensate for the reduction of SRC-1 and CBP in antisense-treated animals. Although our antisense treatment was effective in decreasing coactivator protein expression, as is the case with this technique (76, 77), it did not eliminate expression of the target protein. Finally, there is likely to be a subpopulation of cells containing E2-induced PR that use coactivator-independent mechanisms, as has been proposed in mammary tissue (41). Nevertheless, our data provide evidence that SRC-1 and CBP are important in modulating ER-mediated induction of PR gene expression in a distinct population of cells in the behaviorally-relevant VMN.

E2-induced PR in the VMN are necessary for the expression of progesterone-facilitated sexual behavior in female rodents (21). Exp 1 of the present study demonstrates that SRC-1 and CBP are important in ER-mediated transactivation of PR gene expression in the behaviorally relevant VMN. Therefore, we investigated if these nuclear receptor coactivators function in the VMN to modulate the expression of hormone-dependent reproductive behavior. Indeed, infusion of antisense ODNs to both SRC-1 and CBP mRNA in the VMN of E2- and progesterone-treated female rats decreased the lordosis intensity when compared with rats treated with scrambled ODNs (Table 2). These data extend our findings from Exp 1 and provide further evidence that SRC-1 and CBP influence hormone action in the VMN. In addition, these

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results of coactivator antisense treatment on lordosis are consistent with the concept that nuclear receptor coactivators serve a permissive role in the expression of hormone-dependent sexual behavior. Finally, it should be noted that there are likely to be subpopulations of behaviorally relevant ovarian steroid receptors that operate through coactivator-independent pathways, and thus would not be directly influenced by our experimental design.

The present studies do not distinguish whether these behavioral effects are due to a decrease in coactivator function on the activity of ER, PR, or both. One possibility, as suggested by the results of Exp 1, is that antisense to SRC-1 and CBP mRNA reduces the number of E2-induced PR, resulting in a decrease in the behavioral responsiveness to progesterone in the VMN. In support of this concept, similar behavioral deficits are caused by decreased PR activity in brain using antagonists (78, 79) or antisense to PR (80–82). However, while these other studies detected effects on the lordosis quotient, the present study did not. This discrepancy may be due to compensation by other coactivators (as described above) and/or the difficulty in suppressing coactivator expression, which is more ubiquitous (40, 41, 44, 46) than the temporally defined expression of PR (14, 22, 83). Another possibility for the present effect of antisense on behavior, although not exclusive of the first, is that PR activity is diminished due to the decrease in coactivators, resulting in a reduction in progesterone-facilitated behavior. Additionally, the expression of other estrogen-regulated behaviorally-relevant genes in the VMN (*e.g.* preproenkephalin and oxytocin receptors, see Refs. 84–87) may have been affected by coactivator antisense treatment in our experiments, and thus altered hormone-dependent reproductive behavior. Future experiments on the role of nuclear receptor coactivators in hormone action in brain will need to be done to test these various hypotheses.

Our results, that nuclear receptor coactivators function in ER action in brain, are further supported by findings that SRC-1 is required for the estrogen-dependent development of the sexually dimorphic nucleus of the preoptic area and the development of normal male sexual behavior in rats (45). The present studies provide evidence for an integral role of the nuclear receptor coactivators, SRC-1 and CBP, in hormone-dependent gene expression in brain and the expression of behavior. Reduction of coactivator expression by antisense ODNs decreased ER-mediated PR gene expression in the VMN. Furthermore, we found that nuclear receptor coactivators function in the VMN to modulate hormone-dependent female reproductive behavior. The mechanisms by which hormones act in a tissue-specific manner is a fundamental issue in steroid hormone action. Nuclear receptor coactivator action in brain appears to be an important mechanism for the fine-tuning of steroid responsiveness within individual neurons and the regulation of behavior. In future studies, it will be critical to investigate the function of other coactivators in the modulation of hormone action in brain and behavior.

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