Silencing steroid receptor coactivator-1 in the nucleus of the solitary tract reduces estrogenic effects on feeding and apolipoprotein A-IV expression

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Ling Shen[‡], **Yin Liu**[‡], **Patrick Tso**[‡], **David Q.-H. Wang**[§], **W. Sean Davidson**[‡], **Stephen C. Woods**[¶], **and Min Liu**[‡1]

From the Departments of [‡]*Pathology and Laboratory Medicine and* [¶]*Psychiatry and Behavioral Neuroscience, University of Cincinnati College of Medicine, Cincinnati, Ohio 45237 and the* [§]*Department of Medicine, Division of Gastroenterology and Liver Diseases, Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, New York 10461*

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We previously found that 17β-estradiol (E2) stimulates *apolipoprotein A-IV* (*apoA-IV*) gene expression in the nucleus of the solitary tract (NTS) of lean ovariectomized (OVX) rodents. Here we report that in the NTS of high-fat diet-induced obese (DIO) rats, the *apoA-IV* mRNA level is significantly reduced and that the estrogenic effects on *apoA-IV* gene expression and food intake are impaired. E2 regulates *apoA-IV* gene expression through its nuclear receptor α (ERα), which requires co-activators, such as steroid receptor coactivator-1 (SRC-1), to facilitate the transcription of targeted genes. Interestingly, *SRC-1* gene expression is significantly reduced in DIO OVX rats. SRC-1 is colocalized with apoA-IV in the cells of the NTS and E2 treatment enhances the recruitment of ERα and SRC-1 to the estrogen response element at the apoA-V promoter, implying the participation of SRC-1 in E2's stimulatory effect on *apoA-IV* gene expression. Using small hairpin RNA (shRNA), which was validated in cultured neuronal cells, we found that *SRC-1* gene knockdown specifically in the NTS significantly diminished E2's anorectic action, leading to increased food intake and body weight. More importantly, the stimulatory effect of E2 on *apoA-IV* gene expression in the NTS was significantly attenuated in SRC-1 knockdown rats. These results collectively demonstrate the critical roles of NTS SRC-1 in mediating E2's actions on food intake and *apoA-IV* gene expression and suggest that reduced levels of endogenous SRC-1 and apoA-IV expression are responsible for the impaired E2's anorectic action in obese females.

Estrogens, especially 17β-estradiol (E2),[2] potently facilitate satiation, leading to lower food intake and body weight in many species, including humans. Compelling evidence supports that E2 exerts its catabolic action indirectly through enhancing the strength of other signals implicated in the direct control of food intake and energy expenditure (1), including the satiation factor apoA-IV. Ovariectomy (OVX) in female rodents reduces *apoA-IV* gene expression in the nucleus tractus solitarius (NTS), and this is reversed by cyclic E2 replacement. Additionally, E2 enhances the satiating potency of apoA-IV (2), and more importantly, compared with OVX wildtype controls, OVX apoA-IV knock-out (KO) mice have a blunted reduction of feeding in response to E2, thereby eating significantly more food and gaining more body weight during the period of cyclic E2 replacement (2). These data collectively suggest that increased endogenous apoA-IV signaling may partially mediate E2-induced inhibition of feeding in lean females. Whether or not these effects generalize to high-fat diet (HFD)-induced obesity is unknown.

Obesity is a growing global health problem. One well-established risk factor predisposing to obesity is the amount of fat in the diet. Epidemiological studies have identified a significant positive correlation between average dietary fat intake and the incidence of obesity (3, 4). Recent data suggest that obese animals have blunted satiety, raising the possibility that defective signaling of apoA-IV and an impaired response of *apoA-IV* gene expression to E2 may contribute to the etiology of obesity in females. In the present study, we first addressed the feasibility of these hypotheses in an established rat model of HFD-induced obesity (5), and then asked whether the estrogenic effects on *apoA-IV* gene expression and energy intake are attenuated in the OVX rats after chronic HFD consumption.

Our previous studies demonstrated that E2 regulates *apoA-IV* gene expression through estrogen receptor α (ERα) (6). E2-activated ERα may recruit steroid receptor coactivator-1 (SRC-1), a nuclear receptor coactivator, to DNA promotion sites to facilitate the transcription of targeted genes (7). Interestingly, SRC-1 KO mice are sensitive to diet-induced obesity (8), and the effects of E2 replacement on body weight, food intake, and energy expenditure are blunted in OVX SRC-1 KO mice (9), suggesting that SRC-1 is required for E2's catabolic actions. We therefore performed *in vitro* and *in vivo* studies to determine whether SRC-1 signaling is necessary to mediate E2 effects on *apoA-IV* gene expression and food intake using lentiviral-mediated small hairpin RNA (shRNA), which was specifically delivered into the NTS.

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[1] To whom correspondence should be addressed. Tel.: 513-558-4957; Fax: 513-558-1312; E-mail: lium@uc.edu.

[2] The abbreviations used are: E2, 17β-estradiol; SRC-1, steroid receptor coactivator-1; apoA-IV, apolipoprotein A-IV; OVX, ovariectomy; NTS, nucleus of the solitary tract; shRNA, small hairpin RNA; HFD, high-fat diet; LFD, low-fat diet; ERα, estrogen receptor α; KO, knock-out; PHD, pair HFD-fed; DIO, diet-induced obese; qPCR, quantitative PCR; ERE, estrogen response element; shCTL, control shRNA; GFP, green fluorescent protein.

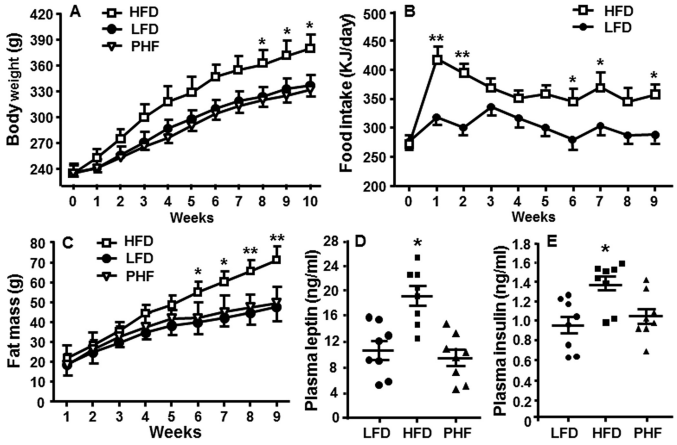

Figure 1. Alteration of metabolic parameters in DIO OVX rats. HFD-fed OVX rats had significantly increased body weight (*A*), daily energy intake (*B*), and fat mass (*C*), compared with LFD-fed OVX controls. Chronic HFD consumption also significantly increased plasma leptin and insulin levels (*D* and *E*). Data represent mean ± S.E., $n = 8$. *, $p < 0.05$; **, $p < 0.01$, compared with LFD-fed rats.

Results

Chronic consumption of HFD increases food intake and body weight in OVX rats

Adult female rats received bilateral OVX through a midline abdominal incision, as we reported previously (2). One week later, the OVX rats were divided into 3 groups. One group was fed a low-fat diet (LFD) and another was fed a HFD for 10 weeks. Because HFD and LFD rats consumed different amounts of energy each day, a 3rd group of rats, called pair HFD-fed (PHF) rats, received the HFD each day, but in an amount limited to the average daily caloric consumption of the rats fed the LFD *ad libitum*, as we reported previously (10). Food intake, body weight, and body composition assessed by EchoMRI were recorded weekly. During week 10, blood was collected, and plasma was assayed for leptin and insulin by ELISA kits.

As depicted in Fig. 1*A*, body weight of the 3 groups of OVX rats differed beginning at 3 weeks. By week 10, HFD-fed rats were significantly heavier than the other two groups ($p < 0.05$). As expected, PHF rats weighed significantly less than HFD-fed rats ($p < 0.05$), and were not different from the LFD-fed rats. The HFD-fed rats consumed significantly more daily energy ($p < 0.05$, Fig. 1*B*) and had significantly more body weight and fat mass than LFD-fed rats ($p < 0.01$, Fig. 1*C*). Consistent with previous reports (11), HFD-fed OVX rats had elevated plasma leptin and insulin ($p < 0.05$), relative to the other two groups (Fig. 1, *D* and *E*).

ApoA-IV mRNA level is significantly reduced in the NTS of DIO OVX rats

To determine whether the *apoA-IV* mRNA level and the response to E2 are altered after chronic HFD consumption, half the rats ($n = 8$) in each group above then received subcutaneous injection of E2 (2 μg) in 100 μl of vehicle every 4th day (mimicking the ovarian cycle of female rats) for 2 cycles, and the other half received vehicle (100 μl of sesame oil) for the same period. The dose of 2 μg of E2 was chosen because it produced plasma E2 levels similar to peak levels occurring during the ovarian cycle in intact rats (12). We selected 2 cycles of E2 treatment because, within such a short period, E2 reduces food intake, but no change of body weight in LFD-fed rats, as we reported previously (2, 12). The injections occurred at 10 a.m. (lights off from 4 p.m. to 4 a.m.). The rats were fasted for 4 h before being sacrificed at the onset of dark on the 2nd day after the final injections, based on the time when E2 exerts its most robust anorexigenic effects in OVX rats (12, 13). The brains were dissected, and the NTS was micropunched for determining *apoA-IV* mRNA levels by quantitative real-time PCR (qPCR) (2, 14).

Relative to the LFD-fed group, HFD-fed rats receiving vehicle (oil) had a significant reduction of *apoA-IV* mRNA level. E2 treatment stimulated *apoA-IV* gene expression in LFD-fed, but not in HFD-fed, rats (Fig. 2*A*). Importantly, E2 treatment significantly increased *apoA-IV* gene expression in PHF-fed rats (Fig. 2*A*), implying that it is the extra calories consumed, but not the HFD itself, which is responsible for the reduction of *apoA-IV* gene expression.

DIO OVX rats have a diminished response in food intake to acute cyclic E2 treatment

To determine whether the reduced apoA-IV expression in the NTS is associated with altered energy intake in the DIO OVX rats, new cohorts of OVX rats were fed HFD and LFD, respectively, for 10 weeks. They were then treated with E2 or oil for 2 cycles as described above. On the 2nd day after the last injections, the rats were fasted for 4 h before dark and their 24-h food intake and body weight were measured. Compared with oil-treated controls, two cycles of E2 treatment significantly suppressed food intake in the LFD-OVX, but not in the DIO OVX rats (Fig. 2*B*), suggesting an impaired response in food intake to the acute E2 treatment in DIO rats. There was no significant difference in body weight between E2- and oil-treated rats either on LFD or HFD (data not shown).

Chronic HFD consumption significantly reduces SRC-1 gene expression in the NTS of OVX rats

To elucidate the underlying molecular mechanisms, we assayed SRC-1 as a possible candidate mediator. Vehicle-treated HFD-fed rats had significantly lower *SRC-1* mRNA than LFD-fed controls. Interestingly, cyclic E2 treatment did not significantly affect *SRC-1* gene expression in either LFD-fed or HFD-fed rats (Fig. 2*C*). No significant difference was found in *SRC-1* mRNA levels between LFD-fed rats and PHF-fed rats with either oil or E2 treatment (Fig. 2*C*).

Colocalization of SRC-1 with apoA-IV in the NTS

We next asked if SRC-1 is colocalizated with apoA-IV in the NTS. Four OVX rats were deeply anesthetized and perfused, as we have reported previously (2, 15). Coronal brainstem sections were cut through the NTS at 30 μm and dual-labeling immunohistochemistry was conducted with anti-SRC-1 and apoA-IV antibodies to localize SRC-1 and apoA-IV expression. Both antibodies were validated previously (9, 15, 16). SRC-1 signals were identified as red fluorescence (Fig. 3*A*), and apoA-IV signals were recognized as green fluorescence (Fig. 3*B*). The immunohistochemical staining revealed that SRC-1 is colocalized with apoA-IV NTS cells (Fig. 3*C*), providing an anatomical

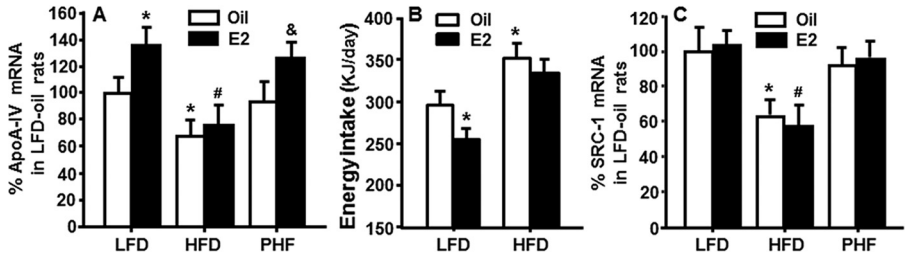

Figure 2. Alterations of gene expression and energy intake in OVX rats on different diets. A, under oil treatment, the apoA-IV mRNA level was significantly reduced in the NTS of HFD-fed OVX rats, compared with LFD-fed OVX rats. E2 treatment increased *apoA-IV* gene expression in both LFD-fed and PHF-fed OVX rats, but not in HFD-fed OVX rats. *B*, comparison of energy intake between LFD- and HFD-fed OVX rats after 2 cycles of E2 or oil treatment. E2 treatment significantly suppressed food intake in LFD-fed OVX, but not in HFD-fed OVX rats, compared with that in oil-treated controls. *C*, HFD-fed OVX rats with oil treatment also had significantly reduced *SRC-1* mRNA levels, compared with LFD-fed and oil-treated OVX rats. E2 treatment did not significantly affect *SRC-1* mRNA levels in either HFD- and LFD-fed OVX rats. No significant difference in *SRC-1* gene expression was found between LFD-fed and PHF-fed OVX rats. Data represent mean ± S.E., $n = 7–8$. *, $p < 0.05$, compared with LFD-fed OVX rats with oil treatment; #, $p < 0.05$, compared with E2-treated LFD-fed rats; and &, $p < 0.05$, compared with oil-treated PHF-fed OVX rats.

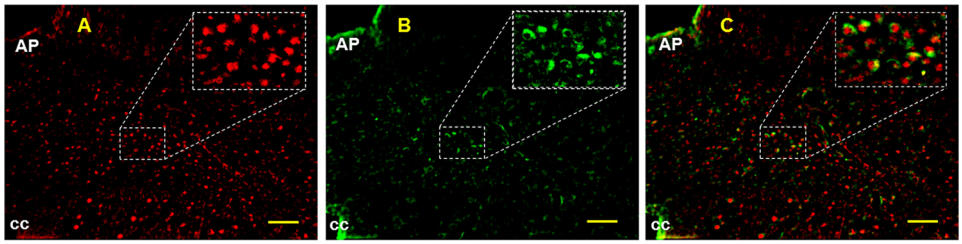

Figure 3. Colocalization of SRC-1 (*A*) and apoA-IV (*B*) in the cells of rat NTS. Using dual immune fluorescent staining, SRC-1 (*A, red*) was found to be colocalized with apoA-IV (*B, green*) in the NTS cells. *Insets* are higher magnification of the region in the *white box*. Sections are representative of four animals in which staining was examined. *AP*, area postrema; *cc*, central canal. *Scale*, 100 μm.

basis for the participation of SRC-1 in E2's regulatory effect on *apoA-IV* gene expression.

E2 enhances the recruitment of ERα and SRC-1 to the estrogen response element (ERE) at the apoA-IV promoter

Although SRC-1 has been reported to form a protein complex with ERα in the hypothalamus in an *in vitro* study (17), it is unknown whether ERα and SRC-1 interact with the ERE located at the 5′-upstream region of the *apoA-IV* promoter in the NTS. We therefore examined this possibility using a sequential biotinylated double-stranded DNA pulldown assay and immunoblot approach, as described previously (18–20). Fourteen OVX rats were divided into two groups ($n = 7$), one receiving E2 (2 μg, subcutaneously) and the other receiving vehicle (sesame oil, 100 μl, subcutaneously) for 2 cycles. On the 2nd day after the last injections, the rats were sacrificed at the onset of dark and the NTS was micropunched.

Two 5′-biotinylated double-stranded 51-mer DNAs that represent *apoA-IV* promoter sequence at 5645 to 5696 were synthesized, one containing an ERE, and the other containing a mutated ERE (6). These double-stranded DNAs were incubated with protein extracts from the NTS for 2 h at 4 °C with constant rotation. The protein–DNA complexes were then immobilized to Streptavidin Dynabeads and separated with a Dynal magnet, according to the manufacturer's instructions (18–20). The beads were washed, resuspended, and boiled in SDS sample buffer for Western blot (6). As depicted in Fig. 4, E2 treatment significantly enhanced the recruitment of ERα and SRC-1 to the ERE located at the apoA-IV promoter, compared with vehicle treatment. No ERα or SRC-1 proteins were detected from the protein extract of the NTS incubated with the biotinylated DNA containing a mutated ERE (Data not shown).

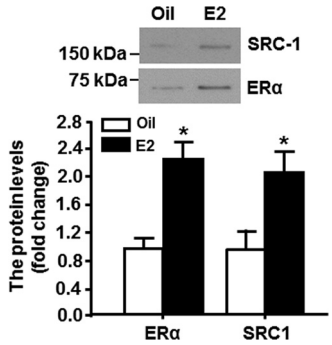

Figure 4. E2 treatment increases the recruitment of ERα and SRC1 to the ERE of the apoA-IV promoter. OVX rats received E2 or vehicle treatment for 2 cycles. The NTS was micropunched. Two biotinylated double-stranded DNAs containing the ERE and mutant ERE, respectively, of the apoA-IV promoter were incubated with protein extracts from the NTS. The protein–DNA complexes were then immobilized to Streptavidin Dynabeads and separated with a Dynal magnet. The beads were washed, resuspended, and boiled in SDS sample buffer. ERα and SRC-1 proteins were detected by immunoblotting. Mean ± S.E., $n = 6–7$. *, $p < 0.05$, compared with oil-treated OVX rats.

shRNA inhibits SRC-1 expression in cultured neuronal cells

To manipulate SRC-1 expression, we used short-hairpin RNA (shRNA), a powerful tool for gene knockdown. From the Viral Vector Core at the University of South Carolina, we obtained three different lentiviral particles with packaged shRNAs that were predicted to target *SRC-1* mRNA, as well as a control lentiviral particle with an empty vector. These particles were transduced into immortalized neuronal cells (CLU213), which express *ERα*, *SRC-1*, and *apoA-IV* genes (6). After 48 h, the transduced cells were selectively grown in a medium containing puromycin (5 μg/ml) for an additional 2 weeks. The cells were treated with E2 (10 nM) for an addi-

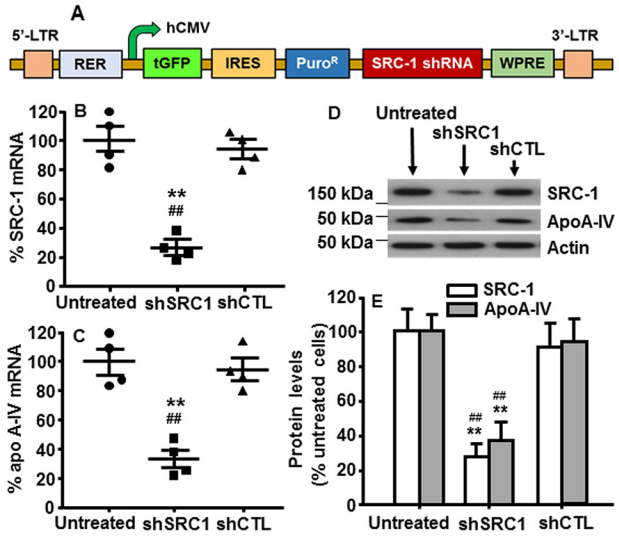

Figure 5. Effects of *SRC-1* gene knockdown induced by shRNA on SRC-1 and apoA-IV expression in E2-treated neuronal cells. *A*, schematic representation of the lentiviral shRNA vector construct. The expression of lentiviral vector containing the shRNA nucleotide is driven by the cytomegalovirus (*CMV*) promoter and the marker gene of turbo green fluorescent protein (*tGFP*) is for visual tracking of transduction and expression. Internal ribosomal entry site (*IRES*) allows expression of tGFP and puromycin resistance genes in a single transcript. Puromycin resistance (*Puro*^{R}) permits antibiotic-selective pressure and propagation of stable integrants. Woodchuck hepatitis post-transcriptional regulatory element (*WPRE*) enhances transgene expression in the target cells. The neuronal cells transduced with SRC-1 shRNA (*shSRC-1*) or shCTL were plated in 24-well plates, treated with E2 (10 nM) for 24 h, and lysed for measuring *SRC-1* and *apoA-IV* mRNA and protein levels by qPCR and Western blotting, respectively. *B* and *C*, the neuronal cells transduced with shSRC-1 had significantly reduced *SRC-1* and *apoA-IV* mRNA levels, compared with the cells transduced with shCTL. *D*, representative immunoblots of SRC-1, apoA-IV, and actin. *E*, quantitative analysis of the protein levels, which were consistent with the changes in their mRNA levels. Mean ± S.E. $n = 4$, **, $p < 0.01$, compared with the untreated group, and ##, $p < 0.01$, compared with the shCTL-transduced cells.

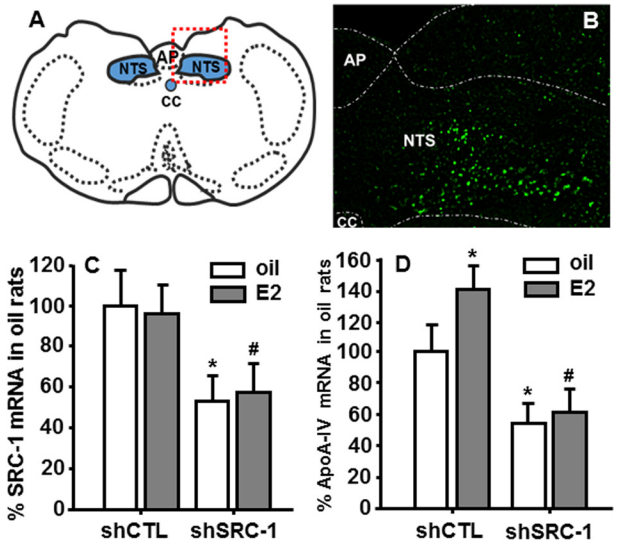

Figure 6. SRC-1 gene knockdown specifically in the NTS of OVX rats. *A*, a schematic drawing for depicting the location in the NTS of rat brain. *B*, a representative micrograph documenting tGFP expression of the lentiviral shNRA in the NTS. *C*, *SRC-1* mRNA levels were significantly reduced in the NTS of rats receiving shSRC-1, relative to that of control rats receiving shCTL. E2 treatment did not significantly affect *SRC-1* mRNA levels in rats receiving either shSRC-1 or shCTL. *D*, SRC-1 knockdown also significantly reduced the *apoA-IV* mRNA level in the NTS, compared with that in rats receiving shCTL. E2 treatment stimulated *apoA-IV* gene expression only in OVX rats receiving shCTL, but not in SRC-1 knockdown OVX rats. Mean ± S.E. $n = 6–7$, *, $p < 0.05$, compared with oil-treated and shCTL-transduced rats, and #, $p < 0.05$, compared with E2-treated and shCTL-transduced rats.

tional 24 h, and then lysed for measuring *SRC-1* and *apoA-IV* mRNA and protein levels by qPCR and Western blotting, respectively.

Among the three SRC-1 shRNAs, the one with the V3LMM_453913 lentiviral vector was selected because of its highest efficiency in the knockdown of SRC-1 expression. Compared with control shRNA (named shCTL), this SRC-1 shRNA (named shSRC-1) significantly reduced *SRC-1* mRNA levels in the neuronal cells (by 75.8%, $p < 0.01$, Fig. 5*B*), consistent with the changes in SRC-1 protein level (69.6% reduction, $p < 0.01$, Fig. 5*E*). No significant difference was found for *SRC-1* mRNA or protein levels between shCTL-transduced cells and untreated neuronal cells.

To determine whether E2's regulation of apoA-IV expression requires SRC-1, we further measured *apoA-IV* mRNA and protein expression in lentiviral shRNAs-transduced neuronal cells by qPCR and Western blotting, respectively. *ApoA-IV* mRNA and protein levels were reduced by 65.2 and 59.2%, respectively ($p < 0.01$), compared with those in shCRL-transduced cells (Fig. 5, *C* and *E*). No significant difference was found in *apoA-IV* mRNA or protein levels between shCTL-transduced cells and untreated neuronal cells. These results indicate that SRC-1 is, at least partially, required for E2's stimulation of apoA-IV expression.

Lentivirus-mediated knockdown of SRC-1 expression in the NTS

To determine the impact of SRC-1 knockdown specifically in the NTS on estrogenic actions on feeding and *apoA-IV* gene expression, OVX rats received bilateral intra-NTS injections of shSRC-1 or shCTL. After a 7-day recovery, the rats with each lentiviral treatment were divided into two subgroups, one receiving E2 (2 μg, subcutaneously), and the other receiving oil (100 μl, subcutaneously) every 4th day for 10 cycles. Body weight and food intake were monitored on the 2nd day after each cycle of E2 or oil treatment. On the 2nd day after the 9th cycle of E2 or oil treatment, the rats were monitored for energy expenditure, as we reported previously (16, 21).

On the 2nd day after the final E2 or oil treatment, 4-h fasted rats were sacrificed and the brains quickly dissected. The brainstem containing the NTS, from −11.8 to −14.1 mm posterior to bregma (22), was sectioned at consecutive intervals of 15 μm (for examining GFP expression in the NTS) and then 200 μm (for the NTS micropunch). All micropunches were snap-frozen in dry ice, prepared for RNA extraction, and the *SRC-1* mRNA level was measured by qPCR.

Animals with correct green fluorescent protein (GFP) location and successful *SRC-1* gene knockdown were included in the data analyses. Fig. 6*A* is a schematic drawing that depicts the location of the NTS of rat brain. Fig. 6*B* is a representative micrograph documenting the expression of GFP of the lentiviral shNRA in the NTS as determined under fluorescence microscopy. *SRC-1* mRNA levels were significantly reduced (by 47.8%) in NTS micropunches of OVX rats receiving shSRC-1, relative to their controls receiving shCTL (Fig. 6*C*). E2 treat-

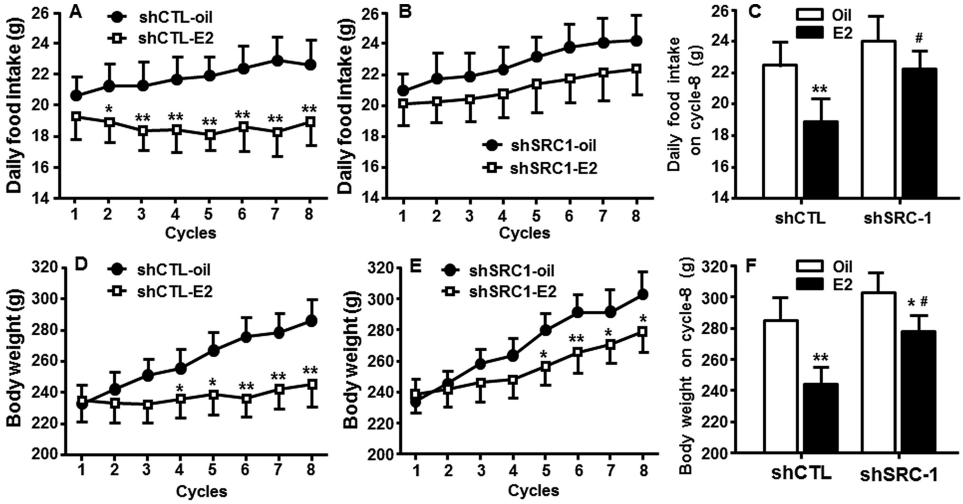

Figure 7. SRC-1 knockdown significantly attenuated E2's actions on food intake and body weight. Time course changes in food intake and body weight were monitored on the 2nd day after each cycle of E2 or oil treatment in shCTL-transduced OVX rats (*A* and *D*) and in shSRC-1-transduced OVX rats (*B* and *E*). The comparison of daily food intake (*C*) and body weight (*F*) was conducted on the 2nd day after the 8th cyclic of E2 or oil treatment in OVX rats. Data are presented as mean ± S.D., $n = 6–7$/group. *, $p < 0.05$; **, $p < 0.01$, compared with oil-treated OVX rats at the same time points; #, $p < 0.05$, compared with E2-treated OVX rats receiving shCTL.

ment did not significantly affect *SRC-1* mRNA levels in either shSRC-1-transduced or shCTL-transduced rats (Fig. 6*C*).

SRC-1 knockdown attenuates E2's effect on apoA-IV gene expression in the NTS

If SRC-1 in the NTS underlies E2's stimulatory effect on *apoA-IV* gene expression through enhancing the efficiency of ERα-activated by E2, gene knockdown of *SRC-1* specifically in the NTS of OVX rats should cause them to be less responsive to E2, compared with their controls. Consistent with this hypothesis, the *apoA-IV* mRNA level was significantly reduced in SRC-1 knockdown rats relative to that in shCTL-transduced rats ($p < 0.05$, Fig. 6*D*). E2 treatment stimulated *apoA-IV* gene expression in shCTL-transduced ($p < 0.05$), but not in shSRC-1-transduced, rats (Fig. 6*D*).

E2's anorectic action is blunted in rats with SRC-1 knockdown in the NTS

It has been reported that a major site for E2 to suppress food intake is the NTS (23–27). Consistent with that, in the OVX rats receiving shCTL, daily food intake was significantly reduced after cyclic treatment of E2, when compared with oil-treated controls (Fig. 7*A*). However, this anorectic action of E2 was diminished in SRC-1 knockdown OVX rats; *i.e.* no difference in food intake between E2 and the oil-treated group was found at any time point (Fig. 7*B*). When comparing food intake on the 2nd day after the 8th cycle of E2 or oil treatment (Fig. 7*C*), a significant difference between SRC-1 knockdown rats and the rats receiving shCTL was found only after E2 treatment (*solid bars*), but not after oil treatment (*open bars*).

Consistent with the changes in food intake, oil-treated OVX rats receiving shCTL had markedly increased body weight, whereas body weight gain was attenuated after cyclic E2 treatment (Fig. 7*D*). Although the growth curve of body weight in SRC-1 knockdown rats was comparable with that in control rats receiving shCTL under oil treatment, cyclic E2 replacement caused much less body weight reduction in SRC-1 knockdown OVX rats (Fig. 7*E*). On the 2nd day after the 8th E2 treatment, the SRC-1 knockdown rats were significantly heavier than that in control rats receiving shCTL (*solid bars*) (Fig. 7*F*).

SRC-1 knockdown in the NTS does not affect energy expenditure

Considering that the E2-induced reduction in body weight gain is partly due to an increase of energy expenditure, which occurs mainly through E2's action at the hypothalamus (28), we also measured energy expenditure. Consistent with previous reports (29, 30). However, no significant difference occurred between shSRC-1- and shCTL-transduced OVX rats under either oil or E2 treatments (Fig. 8, *A–D*). Additionally, no difference in respiratory quotient occurred among the four groups (data not shown). These observations indicate that SRC-1 knockdown in the NTS specifically affects the estrogenic effect on food intake, but not on energy expenditure, leading to a reduction in body weight.

Discussion

In the present studies, we found that the *apoA-IV* mRNA level is significantly reduced in the NTS of HFD-induced obese (DIO) OVX rats. Additionally, the effects on *apoA-IV* gene expression and the reduction of food intake induced by cyclic E2 replacement, which mimics what occurs during the ovarian cycle of female rats (2), was significantly blunted in those DIO rats with OVX. To elucidate the mechanisms underlying the impaired E2 effect on *apoA-IV* expression after chronic HFD consumption, we found that SRC-1, a co-activator of the ERα, is colocalized with apoA-IV in NTS cells and that cyclic E2 treatment enhanced the recruitment of ERα and SRC-1 to the ERE of the apoA-IV promoter. More importantly, the *SRC-1* mRNA level was significantly diminished in the NTS of DIO OVX rats. Using shRNA technology, SRC-1 knockdown significantly reduced the stimulatory effect of E2 on *apoA-IV* expression in cultured neuronal cells. In OVX rats with shRNA-induced SRC-1 knockdown specifically in the NTS, the effects of cyclic

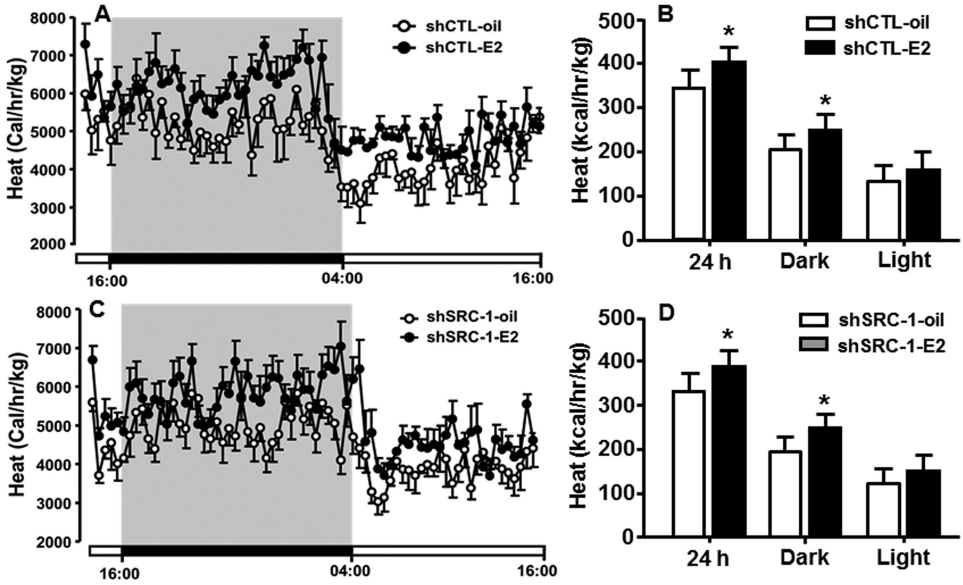

Figure 8. The effect of SRC-1 knockdown on energy expenditure in OVX rats. *A* and *C*, energy expenditure across the 24-h period in E2- or oil-treated OVX rats receiving shCTL and shSRC-1, respectively. *B* and *D*, cumulative energy expenditure during a 24-h period, the dark cycle or the light cycle in E2- or oil-treated OVX rats receiving shCTL and shSRC-1, respectively. Data are presented as mean ± S.D., $n = 6–7$/group. *, $p < 0.05$, compared with oil-treated OVX rats receiving shCTL (*B*) and shSRC-1 (*D*), respectively.

E2 replacement on the reduction of food intake and body weight, as well as the stimulation of *apoA-IV* gene expression, were significantly attenuated. These findings collectively demonstrate that SRC-1 has an important role in mediating E2's actions on feeding as well as upon *apoA-IV* gene expression.

Obesity is a major health problem worldwide. Recent data suggest that obese animals have blunted satiety, raising the possibility that defective signaling of apoA-IV and impaired response of *apoA-IV* gene expression to E2 may contribute to the etiology of obesity in females. Consistent with this hypothesis, we found that *apoA-IV* gene expression in the NTS was significantly decreased in HFD-fed OVX rats, compared with that in either LFD- or HFP-fed OVX rats. More importantly, the stimulatory effect of cyclic E2 replacement on *apoA-IV* gene expression was significantly attenuated.

E2 has potent suppressive effects on food intake and body weight (26). Normal intact female rats eat different amounts of food across their 4-day ovarian cycles. Specifically, the females eat the least during estrus, which occurs right after the preovulatory rise in E2 secretion, and the most during diestrus when E2 levels are lower (12). Disruption of ovarian cycling in rats through OVX leads to increased food intake and body weight gain. However, a cyclic regimen of E2 replacement at a physiologic dose abolishes OVX-induced hyperphagia and normalizes body weight to the levels of gonadal intact rats (2).

E2 acts within the brain to decrease meal size (1), and compelling evidence indicates that the NTS is the critical brain site for E2's anorexigenic action (23, 25, 27). ERα in the dorsal hindbrain is predominately localized in the NTS (31). Administration of a low dose of E2 onto the surface of the hindbrain directly over the caudal NTS significantly decreased food intake and activated ERα-containing neurons in the NTS, without changing the plasma E2 level (27). These observations support our decision to focus on the NTS as the target area to study the impact of chronic HFD consumption on estrogenic actions on feeding and *apoA-IV* gene expression.

It has been well-accepted that E2 acts as an indirect controller of food intake; *i.e.* it inhibits feeding, at least in part, by increasing the potency of satiation factors involved in meal termination (32). Our previous studies have provided evidence that apoA-IV is such a factor. In rats and mice, apoA-IV reduces food intake in a dose-dependent manner after central administration. Blocking its endogenous action with an apoA-IV-specific antibody increases meal size, implying that endogenous apoA-IV plays a role in the tonic inhibition of food intake (33, 34). To determine whether *apoA-IV* gene and protein expression within the NTS are modulated under normal physiological conditions, we compared both *apoA-IV* mRNA and protein levels between male and female rats (6) and found that *apoA-IV* mRNA and protein levels in the NTS of female rats at both diestrous and estrous stages of the ovarian cycle were significantly higher than those in males. The apoA-IV levels in female rats at estrus were significantly higher than that at diestrus, consistent with the preceding increase in circulating E2 levels (35) and higher *ERα* gene expression during the estrus phase (36).

Given the important role of E2 in the regulation of energy homeostasis in the females, OVX should significantly increase food intake and body weight, and suppress *apoA-IV* gene expression in the NTS of female rats. Furthermore, cyclic replacement of E2 should be able to normalize these changes in food intake, body weight, and *apoA-IV* mRNA levels in OVX animals. Consistent with these hypotheses, OVX-induced alterations, including increased food intake and body weight, and decreased *apoA-IV* gene expression in the NTS, were all reversed by cyclic E2 treatment (2).

In our previous studies, we demonstrated that E2 enhances the anorectic potency of apoA-IV in OVX rats. Intra-4th ven-

tricular administration of low doses of apoA-IV reduced food intake more in OVX rats treated with E2 than that in vehicle-treated controls (2). Consistent with this, OVX apoA-IV KO mice had a reduced feeding response to E2, eating significantly more food and gaining more body weight than OVX wildtype mice during the period of cyclic E2 replacement (2). These data indicate that increased signaling by endogenous apoA-IV may partially mediate E2-induced inhibition of feeding.

E2 exerts its physiological function through its receptors, and there is compelling evidence that E2's effect on energy balance is primarily mediated by ERα. ERα KO mice exhibit an obesity phenotype, whereas ERβ KO mice remain lean (37). Deletion of ERα in mice blocks the antiobesity effects of E2 replacement (38). Additionally, administration of an ERα-selective agonist (PPT), but not an ERβ-selective agonist (DPN), promotes hypophagia in OVX rats (13). Given that apoA-IV is involved in E2's anorexigenic action, we therefore hypothesized that E2 stimulates *apoA-IV* gene expression through ERα. This hypothesis was supported by the observation that apoA-IV is colocalized with ERα in the NTS of female rats (2). Using cultured primary neuronal cells isolated from embryonic brainstems of rats, we found that both E2 and PPT (ERα agonist), but not DPN (ERβ agonist), significantly stimulated *apoA-IV* gene expression, compared with vehicle controls. When the neuronal cells were treated with E2 conjugated with bovine serum albumin (E2-BSA), which activates membrane-associated ERs, but does not enter cells due to its large size and charge properties (2), no significant difference in *apoA-IV* gene expression was found between E2-BSA and vehicle-treated cells, implying that a nuclear mechanism with ERα is involved in E2's effect on *apoA-IV* gene expression (2).

E2 binds to ERα, forms dimers, and relocalizes the ERα to the nucleus, resulting in subsequent recruitment of the E2–ERα complex to a palindromic sequence, known as ERE, to regulate target gene expression (39). In our previous studies, using a chromatin immunoprecipitation (ChIP) assay, we demonstrated that an ERE located at the upstream region of rat *apoA-IV* promoter is able to recruit ERα in the NTS of OVX rats, and this recruitment was significantly increased after E2 treatment (6). Furthermore, we confirmed that this ERE is necessary for the response to E2 treatment in the *apoA-IV* promoter with luciferase reporter assay (6).

SRC-1 is a transcriptional coregulatory protein that contains several nuclear receptor interacting domains and has an intrinsic histone acetyltransferase activity. SRC-1 is recruited to DNA promotion sites by ligand-activated nuclear receptors, such as E2-activated ERα. SRC-1, in turn, acylates histones, which makes downstream DNA more accessible to transcription. Therefore, SRC-1 promotes ERα's up-regulation of target gene expression (7, 40). Previous studies have demonstrated that SRC-1 KO mice are sensitive to diet-induced obesity (8). More importantly, the estrogenic effects on body weight and food intake are blunted in OVX SRC-1 KO mice (9), implying that SRC-1 is required for E2's actions on catabolic metabolism. Although these findings from SRC-1 KO mice are interesting, it remains unknown at which brain area(s) SRC-1 mediates E2's catabolic action. In the present study, we found that SRC-1 was abundantly expressed and colocalized with apoA-IV in the NTS. Furthermore, the recruitment of ERα and SRC-1 to the ERE of the apoA-IV promoter was enhanced by the cyclic replacement of E2. These findings provided neuroanatomical and biochemical basis that SRC-1 in the NTS could be involved in effects of E2-ERα signaling on energy intake and *apoA-IV* gene expression.

RNA interference is a cellular mechanism to inhibit expression of a target gene in a highly specific manner (41). shRNA expression has the advantage of providing a continuous source of silencing molecules and is a potent experimental tool for long-term gene silencing (42). With a newly generated shRNA for SRC-1, we validated its efficiency in cultured neuronal cells. Compared with shCTL, SRC-1 shRNA (shSRC-1) significantly reduced *SRC-1* mRNA and protein expression. Interestingly, the SRC-1 knockdown also significantly reduced *apoA-IV* mRNA and protein levels in E2-treated neuronal cells, indicating that SRC-1 is, at least partially, required for E2's stimulation on *apoA-IV* gene expression.

We further determined the functional involvement of SRC-1 in E2's effects on feeding and *apoA-IV* gene expression by directly transducing shSRC-1 or shCTL into the NTS of OVX rats. In control OVX rats receiving shCTL, cyclic E2 treatment significantly reduced daily food intake, compared with oil treatment. However, this anorectic action of E2 was diminished in SRC-1 knockdown OVX rats because no significant difference in food intake between E2- and oil-treated rats was found at any time point. Consistent with the changes in food intake, oil-treated shCTL-transduced OVX rats had markedly increased body weight, and this body weight gain was reduced after cyclic E2 treatment. Although the increase of body weight in SRC-1 knockdown rats was comparable with that in control rats receiving shCTL under oil treatment, cyclic E2 replacement caused much less reduction of body weight in SRC-1 knockdown OVX rats, compared with shCTL rats. By the end of the experiment, SRC-1 knockdown rats were significantly heavier than shCTL-control rats. Interestingly, no significant difference in energy expenditure was found between shSRC-1 knockdown and shCTL-control rats under either oil or E2 treatment, indicating that SRC-1 knockdown in the NTS specifically attenuates the estrogenic effect on energy intake, but not on energy expenditure. The augmented food consumption is responsible for the increase of body weight.

It is noteworthy that knockdown of SRC-1 also reduced basal expression of apoA-IV expression in the NTS, indicating that ERα may not be the only receptor interacting with SRC-1. This could have any of several possible explanations. First, whereas ovariectomy significantly reduces E2 levels, the OVX rats are not absolutely devoid of E2 in the circulation (35), because estrogens can be synthesized in non-reproductive tissue, including liver, heart, muscle, and adipose tissue (43). The low level of endogenous E2 may still be able to stimulate *apoA-IV* gene expression to a certain degree through the interaction with SRC-1. Therefore, SRC-1 knockdown will attenuate endogenous E2's effect in OVX rats, leading to reduced basal expression of apoA-IV. Second, our previous studies have found that central *apoA-IV* gene expression is up-regulated by leptin through the JAK/STAT3 signaling pathway (15). The transcriptional activation by STAT3 has been reported to

require the recruitment of SRC-1, which functions as a coactivator of STAT3 protein (44). SRC-1 knockdown may therefore impair the effect of endogenous leptin signaling on *apoA-IV* gene expression, leading to reduced basal levels of apoA-IV. Third, SRC-1 may serve as a transcription factor for other receptors or signaling pathways, for example, SRC-1 could interact with the p50 subunit and coactivate nuclear factor κB-mediated transactivation (45). Although these questions are beyond the scope of the current studies, determining whether SRC-1 mediates the actions of leptin and/or cytokines on *apoA-IV* gene expression in diet-induced obese animals will be investigated in the future.

In summary, we have demonstrated that SRC-1 in the NTS is a physiological mediator of E2's anorectic action, and provided insight into the underlying molecular mechanisms as to where and how E2 inhibits food intake without influencing energy expenditure. It is known that women, after entering menopause, are at increased risk for developing the metabolic syndrome. Hormone replacement therapy can curb postmenopausal weight gain but also increase the risk of heart disease and breast cancer (46). One solution to this dilemma would be to target the downstream genes of E2 signaling that are involved in energy balance without causing unwanted estrogenic side effects. Thus, identifying altered key components, such as SRC-1, which mediate E2's effect on *apoA-IV* gene expression in OVX rats, will reveal novel pharmacological targets for reducing post-menopausal obesity risk without deleterious side effects.

Experimental procedures

Animals

Adult female Long-Evans rats (Harlan, Indianapolis, IN) were individually housed in a temperature-controlled vivarium on a 12-h light/dark cycle. Rodent chow (Teklad; Harlan) and water were provided *ad libitum* except where noted. All animal procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Animals and approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Materials and diets

E2 and sesame oil (as vehicle) were purchased from Sigma. Antiserum against rat apoA-IV was raised from goat and characterized as described previously (47). Mouse monoclonal antibody against ERα (catalog number PIMA513304), Dynabeads™ M-280 Streptavidin (catalog number 11205D), and poly(dI-dC) (catalog number 20148E) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Rabbit monoclonal antibody against SRC-1 (catalog number 2191) was purchased from Cell Signaling (Danvers, MA). Rat hypothalamic neuronal cells (CLU213) were obtained from Cedarlane Laboratories (Burlington, NC). Cell culture medium was from Invitrogen. All other chemicals were purchased from Sigma. Two pelleted semi-purified (AIN-93M), nutritionally complete experimental HFD and LFD diets were prepared by Dyets (Bethlehem, PA), as reported previously (5).

Double-labeling immunohistochemistry

The rats were transcardially perfused with 0.9% saline, followed by 4% sodium phosphate-buffered paraformaldehyde. Brains were postfixed for an additional 4 h, stored in 15% and then 30% sucrose solution at 4 °C. Free-floating coronal brainstem sections (30 μm) were cut through the NTS on a microtome (Leica, Buffalo Grove, IL) (2). After washing, the sections were blocked with 5% normal donkey serum in PBS containing 0.3% Triton X-100 for 1 h. Then, sections were incubated with rabbit monoclonal antibody against SRC-1 (1:1000) and goat anti-apoA-IV antibody (1:400) overnight at 4 °C. Secondary antibodies were used as appropriate, including 1:500 diluted donkey anti-rabbit (for SRC-1) or donkey anti-goat (for apoA-IV) IgG conjugated to fluorescein, Alexa Fluor 594, or Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR), respectively, for 1 h at room temperature. Fluorescence images were taken using a IX83 fluorescence microscope with *cellSens-Life Science Imaging Software* (Olympus, Waltham, MA). Omission of the primary antibodies as well as substituting the primary antibody with normal rabbit serum or normal goat serum were used to determine the specificity of the antibodies, as we have done previously (15).

DNA pulldown assays and Western blotting

The 5′-biotinylated double-stranded DNA (5′- TCAGAGCAAGACGAACAAGGGGCAGCATGACCCAGTTCCCATCAGCTCTGC-3′) corresponding to position 5645 to 5696 at the 5′-upstream region of the *apoA-IV* promoter and containing the ERE (6) were synthesized by Integrated DNA Technologies (Coralville, IA). The biotinylated DNA containing a mutated ERE (5′-TCAGAGCAAGACGAACAAG*GTTGATGCAGCTAT*CAGTTCCCATCAGCTCTGC-3′), in which the conserved nucleotides of the ERE are replaced (6), as indicated by italic characters, was also synthesized.

The NTS tissues were homogenized in binding buffer (50 mM Hepes, pH 7.3, 2 mM EGTA, 2 mM $MgCl_2$, 1 mM EDTA, 15 mM NaF, 10 mM β-glycerophosphate, 10% glycerol, 100 mM KCl, 1 mM DTT, 10 μg/ml of aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40). After centrifugation, the supernatant were incubated with 10 pmol of the biotinylated double-stranded DNAs and 10 μg/ml of poly(dI-dC), a competitor for nonspecific DNA-binding proteins, for 2 h at 4 °C. After that, 100 μg of Dynabeads M-280 Streptavidin was added and incubated for another 30 min at room temperature, according to the manufacturer's instructions (18, 19). The beads were washed three times with washing buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA), resuspended, and boiled in SDS sample buffer. The bound proteins were detected by Western blot.

Lentiviral constructs

The rat SRC-1 microRNA-adapted shRNA set, including three individual clones (catalog numbers V2LMM_14018, V3LMM_453913, and V3LMM_453915), was obtained from GE Dharmacon (Lafayette, CO) and used to generate lentiviral particles. These three lentiviral constructs contain unique double-stranded shRNA targeting positions +1312, +4218, and +2661, respectively, in the *SRC-1* gene. The shRNA nucleotide sequences specific to SRC-1 messenger RNA (mRNA) were as

follows: 5′-TATGAATCTATAGGATGGG (V2LMM_14018), 5′-TGACTGAACACATTGTTGT (V3LMM_453913), and 5′-TTCTTCTCCACTTTGACCT (V3LMM_453915).

All lentiviral particles were prepared by the Viral Vector Core at the University of South Carolina (48–50) using an approach described previously (48) by transiently transducting 107 293T cells with 15 μg of vector cassette, 10 μg of packaging cassette, and 5 μg of envelope cassette. All vectors were pseudotyped with the vesicular stomatitis virus-glycoprotein envelope cassette. Titers of the vectors were determined by p24 gag enzyme-linked immunosorbent assay. The absence of replication-competent retroviruses was determined by three independent methods: tat transfer assay, vector rescue assay, and p24 gag enzyme-linked immunosorbent assay, as described previously (51). For *in vitro* and *in vivo* studies, titers of 1×10^7 infection units (IU)/ml and 5×10^9 IU/ml were used, respectively. All experimental procedures were approved by the University of Cincinnati Institutional BioSafety Committee.

Neuronal cell culture and transduction

Because no brainstem neuronal cell line is available commercially, we used rat hypothalamic cells (CLU213), which express ERα, SRC-1, and apoA-IV. Those neuronal cells were seeded in HyClone Dulbecco's modified Eagle's medium/high glucose media (with L-glutamine and L-glucose; Thermo Scientific) and 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). Cells were treated with trypsin (Invitrogen) and transferred to 6-well tissue-cultured plate such that they reached 70% confluence overnight. Medium was then removed and replaced with fresh medium with one of the four different lentiviral particles (final concentration 5×10^5 IU/ml). After 48 h, the transduced cells were selectively grown in medium containing puromycin (5 μg/ml) for an additional 2 weeks. The neuronal cells transduced with lentiviral particles were then treated with E2 (10 nM) for an additional 24 h, and lysed for measuring *SRC-1* and *apoA-IV* mRNA and protein levels by qPCR and Western blotting, respectively (2).

Stereotaxic surgery in OVX rats

Overnight-fasted OVX rats were divided into two weight-matched groups ($n = 14$/group). One group received bilateral intra-NTS injections with shSRC-1 (5×10^9 μg/ml, 0.5 μl/site), and the other group received shCTL as control. The coordinates for the NTS were anteroposterior from bregma −13.6 mm, mediolateral ±0.7 mm, and dorsoventral −8.0 mm, based on the atlas of Paxinos and Watson and previous reports (22, 36). To reduce potential tissue damage, each NTS injection was made with a Hamilton syringe (5 μl, model: 75-RN) via an Intergrated Stereotaxic Injector (*iSi*^{TM}) system (Stoelting Co., Wood Dale, IL) at a rate of 0.1 μl/min for 5 min with a 10-min pause between sides (52).

Cyclic E2 or oil treatment in the OVX rats transduced with lentiviral particles

The OVX rats receiving shSRC-1 or shCTL injections were divided into two weight-matched groups on day 7 after surgery. One group was injected with E2 (2 μg/100 μl if sesame oil, subcutaneously, every 4th day) for 10 cycles, and the other group was injected with the same volume of sesame oil for the same period. Injections were done between 9 and 10 a.m. Food intake and body weight were monitored on the 2nd day after each cycle of E2 or oil treatment.

Indirect calorimetry

A continuous monitoring system (Oxymax Equal Flow System, Columbus OH) was used to determine energy expenditure during the 9th cycle of E2 or oil treatment. Rats from each group were placed in the system for 48 h. The first 24 h were considered adaptation and the data from the next 24 h were analyzed. Data for indirect calorimetry analysis were sampled every 30 min (16).

qPCR for apoA-IV mRNA measurement

Total RNA was extracted from the cultured neuronal cells or the tissues from rat NTS using a PureLink RNA Mini Kit (Ambion-Life Technologies). RNA was quantified using a NanoDrop 2000 (Thermo Scientific). Then, 250 ng of RNA from each sample was used for reverse transcription to cDNA with a Transcriptor First Strand cDNA Synthesis Kit (Roche Molecular Diagnostics). *ApoA-IV* mRNA levels were quantified by qPCR using TaqMan Fast Advanced Master Mix with TaqMan Gene Expression Assay for *apoA-IV* with a StepOne^{TM} Plus device (Thermo Scientific). 45 S mRNA levels from each sample were used as internal controls to normalize the mRNA levels, as described previously (53).

Western blot analysis

The Western blot analysis was performed with the following antibodies: anti-ERα (1:1000), anti-SRC-1 (1:1000), anti-apoA-IV (1:3000), as previously described (6, 15).

Measurement of plasma samples

Blood samples were taken from the tail vein, and plasma leptin and insulin were measured with ELISA kits (Millipore) (16).

Statistical analysis

All data are presented as mean ± S.E. Data were analyzed using parametric statistics (SigmaPlot version 12.0). *In vitro* experiments were carried out in triplicate and performed on 3–4 separate occasions. Differences among more than two groups were determined using one-way or two-way repeated measures analysis of variance followed by Student-Newman-Keuls test for comparison between treatments. p values less than 0.05 were considered statistically significant.

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