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Receptor Phosphorylation Mediates Estradiol Reduction of α_2-Adrenoceptor Coupling to G Protein in the Hypothalamus of Female Rats

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Estrogen increases evoked norepinephrine release in the hypothalamus of female rodents, in part by reducing the ability of α_2-adrenoceptors to act as negative feedback inhibitors of norepinephrine release. Estrogen enhancement of norepinephrine release in the hypothalamus correlates with decreased coupling of the α_2-adrenoceptor to G protein. To determine the mechanism by which estrogen uncouples α_2-adrenoceptors from G protein, we tested the hypothesis that estrogen increases α_2-adrenoceptor phosphorylation. Short-term activation of endogenous serine/threonine phosphatases with protamine or treatment with exogenous phosphatase restored α_2-adrenoceptor coupling to G protein to control levels in hypothalami from estrogen-exposed female rats. Additional experiments examined whether estrogen alters G protein–coupled receptor kinase expression or activity or serine/threonine phosphatase activity. These proteins are involved in G protein–coupled receptor phosphorylation, internalization, and recycling. Estrogen exposure reduced G protein–coupled receptor kinase mRNA, protein, and activity in the hypothalamus. Furthermore, estrogen treatment reduced serine/threonine phosphatase activity in the hypothalamus. Analysis of ligand binding in subcellular fractions demonstrated that estrogen decreases the fraction of internalized α_2-adrenoceptors in the hypothalamus.Therefore, estrogen promotes norepinephrine release in the hypothalamus by stabilizing α_2-adrenoceptor phosphorylation, uncoupling the receptor from G protein. Estrogen may stabilize α_2-adrenoceptor phosphorylation by inhibiting receptor internalization and dephosphorylation.

Key Words: Estrogen; α_2-adrenoceptors; hypothalamus; reproduction; desensitization.

Introduction

Ovarian steroids play a key role in the coordination of reproductive function in female mammals. For example, estrogen is required for normal release of gonadotropin and reproductive behavior in female rodents *(1–3)*. One way estrogen promotes female reproductive behavior is by elevating stimulus-induced release of norepinephrine in the hypothalamus *(1)*. α_2-Adrenoceptors in the hypothalamus act as presynaptic autoreceptors to inhibit the release of norepinephrine *(4)*. Therefore, attenuation of α_2-adrenoceptor inhibition of release of norepinephrine should enhance the release of norepinephrine in the hypothalamus. Indeed, we have shown that estrogen treatment for 2 d decreases α_2-adrenoceptor inhibition of release of norepinephrine in the hypothalamus *(4)*, possibly by decreasing the percentage of α_2-adrenoceptors coupled to G protein *(5,6)*. The mechanism by which estrogen promotes α_2-adrenoceptor uncoupling from G protein in the hypothalamus is not known. However, estrogen appears to act over a similar time course to attenuate β-adrenoceptor function in the hypothalamus by stabilizing the receptor in a phosphorylated state *(7)*.

Phosphorylation of α_2-adrenoceptors on the third intracellular loop and carboxy terminus by serine/threonine kinases uncouples receptors from G protein. G protein–coupled receptor kinase (GRK), protein kinase C (PKC), and protein kinase A (PKA) can all phosphorylate α_2-adrenoceptors and uncouple them from G protein *(8)*. Notably, studies on α_1-adrenoceptors suggest that inhibition of serine/threonine phosphatase activity also promotes receptor phosphorylation and uncoupling from G protein *(9)*. Therefore, either elevation of serine/threonine kinase activity or suppression of serine/threonine phosphatase activity could increase α_2-adrenoceptor phosphorylation, uncoupling the receptor from G protein.

Changes in receptor–G protein coupling are also often associated with changes in subcellular localization of the receptors. In agonist-mediated receptor desensitization, GRK phosphorylates an agonist-occupied receptor and promotes β-arrestin binding to the receptor, uncoupling the receptor from G protein *(10)*. Binding of β-arrestin to a G protein–coupled receptor also initiates receptor internalization *(11)*. PKC and PKA-mediated receptor phosphorylation and un-

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coupling may also be associated with receptor internalization *(12,13)*. Furthermore, several researchers have suggested that without internalization, receptor dephosphorylation and recycling back to the cell surface may not occur *(14,15)*. Endocytotic vesicles, which contain internalized receptors, are associated with high levels of phosphatase activity *(16–18)*. Thus, inhibition of receptor internalization could promote receptor uncoupling from G protein by reducing the rate of receptor dephosphorylation.

The present studies examined whether estrogen uncouples α_2-adrenoceptors from G protein by altering receptor phosphorylation or internalization. Activation of serine/threonine phosphatases in hypothalamus slices or membranes from estrogen-treated animals restored α_2-adrenoceptor–G protein coupling to control levels. The same estrogen treatment that uncoupled α_2-adrenoceptors from G protein also reduced serine/threonine phosphatase activity in the hypothalamus by about 25%. Furthermore, quantitation of α_2-adrenoceptor binding density in vesicular and plasma membrane fractions revealed a decrease in receptor internalization in estrogen-exposed hypothalamus. Therefore, estrogen appears to uncouple α_2-adrenoceptors from G protein in the hypothalamus by stabilizing receptor phosphorylation and inhibiting receptor internalization.

Results

Estradiol Reduces High-Affinity Binding of Norepinephrine to α_2-Adrenoceptors

Initially, we verified that estrogen treatment (2 μg of estradiol benzoate (EB) 48 and 24 h prior to killing) alters the coupling of α_2-adrenoceptors to G protein in the hypothalamus of ovariectomized (OVX) female rats by examining norepinephrine displacement of [^{3}H] RX821002 binding *(5)*. Norepinephrine displaced [^{3}H] RX821002 in hypothalamus membranes in a biphasic fashion, comprising an agonist high-affinity site ($pK_{iH} = 6.9 \pm 0.2$ for control and $pK_{iH} = 6.8 \pm 0.3$ for estrogen-exposed; $n = 4$) and an agonist low-affinity site ($pK_{iL} = 5.4$ for control and $pK_{iL} = 5.5 \pm 0.3$ for estrogen-treated; $n = 4$). In hypothalamus from control animals, α_2-adrenoceptor binding was primarily high affinity with 95% high and 5% low affinity. In agreement with our earlier work *(5)*, agonist affinity in hypothalamus from animals exposed to estrogen for 48 h shifted to 49% high and 51% low affinity. Norepinephrine displacement of [^{3}H] RX821002 binding in preoptic area (POA) membranes was monophasic; α_2-adrenoceptors were all in the agonist high-affinity state independent of the hormonal condition ($pK_{iH} = 6.8 \pm 0.1$ for control and 6.9 ± 0.1 for estrogen-treated; $n = 4$). Treatment with the nonhydrolyzable guanosine 5'-triphosphate analog 5'-guanylylimidodiphosphate (Gpp[NH]p) shifted all α_2-adrenoceptors to the agonist low-affinity state regardless of brain region or hormonal status of the animal (data not shown).

UK 14304 is an agonist that binds selectively to the high-affinity form of the α_2-adrenoceptor, which represents receptor coupled to G protein *(19)*. As observed previously *(5)*, exposure of OVX female rats to estrogen for 48 h reduced [^{3}H] UK 14304 binding by about 65% in hypothalamus membranes ($p < 0.05$; Table 1). By contrast, estrogen treatment did not alter α_2-adrenoceptor high-affinity binding in the POA. There was a trend for estrogen exposure to decrease [^{3}H] UK 14304 binding in the hypothalamus after 24 h (control = 132 ± 16 fmol/mg; EB = 92 ± 14 fmol/mg; $n = 4$), but this effect was not statistically significant.

Effects of Phosphatase Activation on α_2-Adrenoceptor High-Affinity Binding

Receptor phosphorylation shifts the affinity for agonists from high to low affinity, reflecting receptor–G protein uncoupling *(15,20)*. Therefore, we examined whether phosphatase activation can return α_2-adrenoceptor high-affinity binding to control levels in membranes from estrogen-treated animals. Earlier studies *(17,18)* suggest that dephosphorylation of receptors is mediated by a protamine-activated phosphatase 2A (PP2A). Therefore, we pretreated hypothalamus and POA slices from OVX control and EB-treated female rats with 200 μg/mL protamine for 30 min to activate PP2A. [^{3}H] UK 14304 binding density in hypothalamus membranes from EB-treated animals returned to control levels when slices were treated with protamime (Table 1). Treatment with protamine had no effect on [^{3}H] UK 14304 binding in hypothalamus membranes from OVX control rats or in POA membranes from OVX control and estrogen-exposed rats.

To further implicate phosphorylation in estrogen-induced reductions in [^{3}H] UK 14304 binding, we pretreated hypothalamus and POA membranes for 2 h at 37°C with 100 U/mL of alkaline phosphatase. Estrogen-induced reductions in [^{3}H] UK 14304 binding in hypothalamus membranes were still apparent when membranes were preincubated at 37°C for 2 h ($p < 0.05$; Table 2). Treatment with alkaline phosphatase returned [^{3}H] UK 14304 binding in membranes from estrogen-exposed hypothalamus to control levels (Table 2). Treatment with alkaline phosphatase had no effect on [^{3}H] UK 14304 binding in hypothalamus membranes from OVX control rats or in POA membranes from OVX control or estrogen-treated rats (Table 2).

Estradiol Reduces GRK 2 mRNA, Protein, and Activity

Our data suggest that estradiol-induced uncoupling of α_2-adrenoceptors from G protein in the hypothalamus can be reversed by dephosphorylation. Thus, we determined whether estrogen regulates GRKs, serine/threonine kinases that phosphorylate, uncouple, and desensitize receptors. Because GRK 2 is highly expressed in rat brain *(21)*, we examined GRK 2 mRNA and immunoreactivity in the POA and hypothalamus. Immunoreactivity for GRK 2 in the hypothalamus significantly decreased 24 h after injection of

Table 1
Effects of Phosphatase Activation on High-Affinity
α_2-Adrenoceptor Binding in Membranes Prepared From Brain Slices[a]

	[^{3}H]-UK 14304 binding density (fmol/mg, X ± SEM)			
	Vehicle treated		Protamine treated	
Tissue	Control	EB	Control	EB
Hypothalamus	128.5 ± 22.4	45.6 ± 11.6[b]	103.6 ± 13.8	113.3 ± 20.6
Preoptic area	133.3 ± 41.5	140.3 ± 28.8	93.5 ± 20.5	87.7 ± 18.2

[a]Scatchard analysis of [^{3}H] UK 14304 binding was conducted on membranes from hypothalamus or preoptic area slices treated with vehicle or protamine (200 µg/mL) for 30 min. All data are means ± SEM of three to six independent replications.
[b]$p < 0.05$ vs all other groups.

Table 2
Effects of In Vivo Estrogen Exposure
and Phosphatase Treatment of Membranes on High-Affinity α_2-Adrenoceptor Binding[a]

	[^{3}H]-UK 14304 binding density (fmol/mg, X ± SEM)			
	Vehicle treated		Alkaline phosphatase treated	
	Control	EB	Control	EB
Hypothalamus	89.0 ± 2.0	66.9 ± 3.7[b]	85.1 ± 3.8	95.0 ± 3.3
Preoptic area	79.9 ± 16.4	83.2 ± 9.8	80.0 ± 8.5	74.9 ± 7.1

[a]Scatchard analysis of [^{3}H] UK 14304 binding was conducted on hypothalamus and preoptic area membranes treated with vehicle or 100 U/mL of alkaline phosphatase for 2 h at 37°C. All data are means ± SEM of three to six independent replications.
[b]$p < 0.05$ vs all other groups.

2 µg of EB ($p < 0.05$; Fig. 1A,B). By contrast, GRK 2 immunoreactivity in the POA did not change significantly after 24 or 48 h of exposure to EB (Fig. 1B).

Northern blots were used to determine whether changes in GRK 2 protein expression are accompanied by changes in GRK 2 mRNA expression. As previously shown *(21)*, the GRK 2 cDNA fragment corresponding to nucleotides 808–1006 of the rat cDNA detected a 3.8-kb transcript located slightly below the signal for the 28S rRNA. Figure 1C,D demonstrates an approx 50% decrease in GRK 2 mRNA expression in the hypothalamus 24 h after treatment with EB ($p < 0.05$; Fisher). GRK 2 mRNA levels were still significantly below OVX control levels in the hypothalamus 48 h after treatment with estrogen ($p < 0.05$). Reverse transcriptase polymerase chain reaction experiments confirmed the Northern data, detecting a significant decrease in GRK 2 mRNA at 24 h ($p < 0.05$; Fisher) and a trend for a decrease at 48 h after treatment with EB (data not shown).

To determine whether changes in GRK 2 immunoreactivity and mRNA expression correlate with changes in enzyme activity, GRK catalytic activity was examined in the hypothalamus and POA. Figure 2A shows that GRK activity in cytosolic fractions of the hypothalamus decreased by 36% 24 h after treatment with EB ($p < 0.05$; Fisher). GRK activity in the hypothalamus tended to remain suppressed 48 h after the administration of estrogen, but the reduction was no longer significant. By contrast, GRK activity did not change in the POA following the administration of estradiol. As shown in Fig. 2B, treatment with EB had no effect on membrane GRK activity in the hypothalamus or POA.

Estrogen Reduces Phosphatase Activity

Because estrogen appears not to elevate α_2-adrenoceptor phosphorylation by increasing GRK activity in the hypothalamus, we examined estrogen effects on cytosolic serine/threonine phosphatase activity in the hypothalamus and POA. Figure 3 shows that estrogen treatment for 48 h significantly decreased phosphatase activity by approx 25% in the hypothalamus ($p < 0.05$). Treatment with estrogen did not alter phosphatase activity in the POA (Fig. 3).

Estradiol Decreases α_2-Adrenoceptor Internalization

Receptor phosphorylation is often followed by receptor internalization *(12)*. We determined whether treatment with EB modifies α_2-adrenoceptor subcellular localization by evaluating [^{3}H] RX821002 binding in light-vesicle and plasma membrane fractions from POA and hypothalamus. Fraction purity was determined by immunoblotting for carboxy-

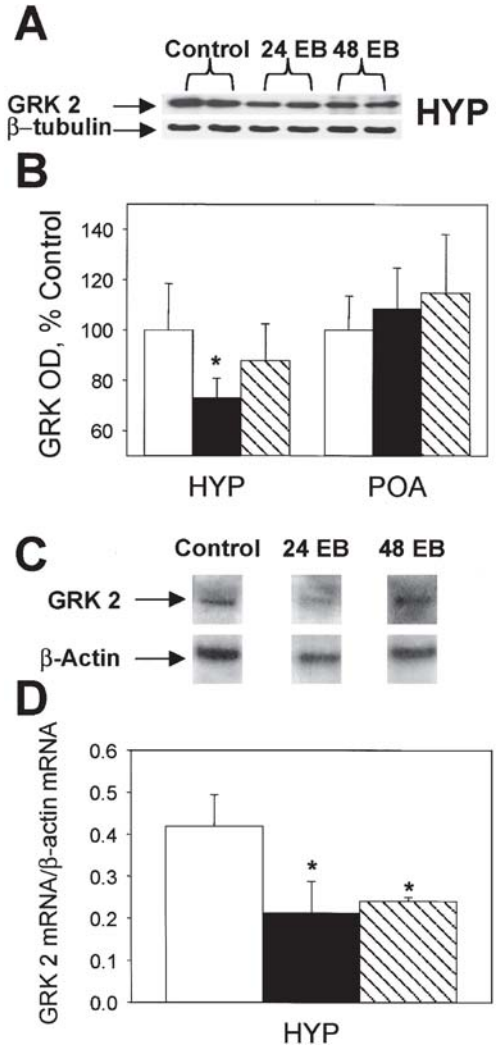

Fig. 1. Regulation of GRK 2 protein and mRNA by estradiol. (**A**) Representative immunoblots using GRK 2 antibody. OVX female rats received sc injections of 0.1 mL of vehicle 24 and 48 h prior to dissection (Control), 2 µg of EB 24 h prior to dissection (24 EB), or 2 µg of EB 48 and 24 h prior to dissection (48 EB). (**B**) Quantitative analysis of GRK 2 protein in 25 µg of whole-cell extract from hypothalamus (HYP) and POA. , Control; ■, 24 EB; ⊠, 48 EB. Data are expressed as a percentage of the OD of the control treatment, calculated as OD of the GRK 2 signal corrected by the OD of the β-tubulin signal from the same sample. All data are means ± SEM of four to six independent replications. *$p < 0.05$ vs Control. (**C**) Representative Northern blots for GRK 2 mRNA. All data presented came from a single autoradiogram. (**D**) Quantitative analysis of Northern blots for GRK 2 mRNA in 10 µg of total RNA from HYP. Data are expressed as OD of the GRK 2 signal corrected by the OD of the β-actin signal from the same sample. All data are means ± SEM of three to five independent replications. *$p < 0.05$ vs Control.

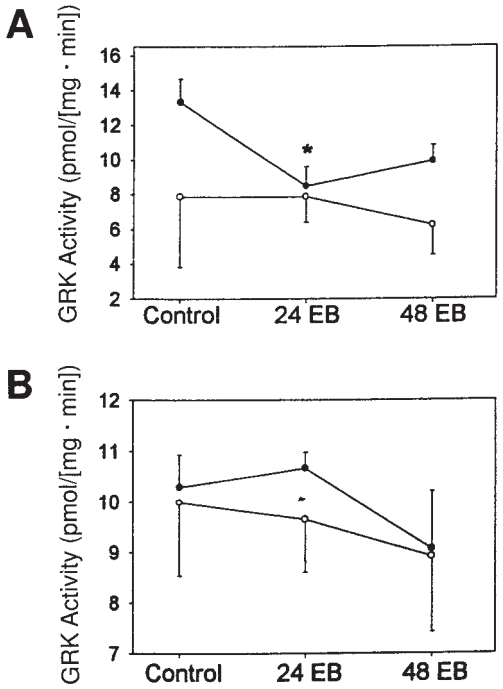

Fig. 2. Regulation of GRK activity by estradiol. (**A**) Estrogen regulation of GRK activity in the cytosolic fraction. OVX female rats received sc injections of 0.1 mL of vehicle 24 h prior to dissection (Control), 2 µg of EB 24 h prior to dissection (24 EB), or 2 µg of EB 48 and 24 h prior to dissection (48 EB). Ten micrograms of HYP (●) or POA ( ) protein was assayed as described in Materials and Methods. All points are means ± SEM of four to six independent replications. *$p < 0.05$ vs Control. (**B**) Estrogen regulation of GRK activity in the membrane fraction. Ten micrograms of HYP (●) or POA ( ) protein was assayed as in (A). All points are means ± SEM of three to six independent replications.

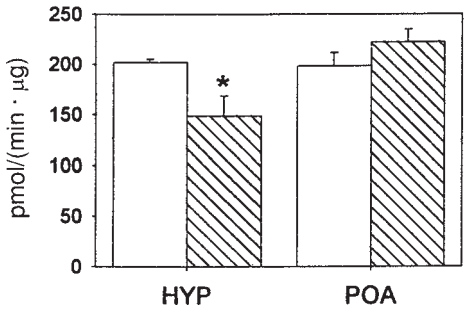

Fig. 3. Estrogen regulation of serine/threonine phosphatase activity. Five micrograms of hypothalamus (HYP) or POA cytosolic protein was incubated for 5 min with PKC-labeled protamine at 25°C. , Control; ⩓, 48-h EB. All points are means ± SEM of four independent replications. *$p < 0.05$ vs Control.

peptidase E (vesicular enzyme) and SNAP-25 (plasma membrane-enriched, vesicle-trafficking protein), and by radioligand binding ([^{3}H] ouabain) for Na^+/K^+ ATPase (plasma membrane marker). Plasma membrane fractions exhibited a 10-fold higher density of [^{3}H] ouabain binding than light-vesicle fractions, strong immunostaining for SNAP-25, and no visible immunostaining for carboxypeptidase E. Light-vesicle fractions showed strong immunostaining for carboxypeptidase E, very light immunostaining for SNAP-25, and very low levels of specific [^{3}H] ouabain binding (data not shown).

As reported previously *(6,22)*, treatment with EB for 48 h modestly but significantly increases [^{3}H] RX821002 binding density by about 30% in plasma membranes from the

Table 3
Effects of Estrogen on α_2-Adrenoceptor-Binding Sites in Light-Vesicle and Plasma Membrane Fractions[a]

	B_{max} (fmol/mg, $X \pm$ SEM)			
	Light vesicle	Plasma membrane	Light vesicle (%)	Plasma membrane (%)
Hypothalamus				
Control	49.1 ± 10.3	197.3 ± 13.9	15.9 ± 1.8	84.1 ± 1.8
EB	49.3 ± 10.5	257.7 ± 28.3[b]	10.0 ± 1.3[b]	90.0 ± 1.3[b]
Preoptic area				
Control	40.3 ± 10.5	299.4 ± 22.3	12.2 ± 4.2	87.8 ± 4.2
EB	43.3 ± 15.6	283.2 ± 14.7	12.3 ± 3.7	87.7 ± 3.7

[a]Saturation analysis of [^{3}H] RX821002 binding was carried out on membrane fractions from OVX female rats treated with vehicle (control) or 2 μg of EB injected 48 and 24 h prior to dissection (EB). The percentage in each fraction was calculated only when data were available from both light vesicles and plasma membranes. Hormonal status of the animal had no effect on [^{3}H] RX821002 (2.7–8.2 n*M*) binding affinity regardless of brain region or subcellular fraction assayed. All data are means ± SEM of three to five independent replications for B_{max} or three independent replications for percentage.
[b]$p < 0.05$ vs control.

hypothalamus ($p < 0.05$; Table 3). Estrogen did not affect the absolute level of [^{3}H] RX821002 binding in hypothalamus light vesicles. However, because approx 85% of the α_2-adrenoceptor localized to the plasma membrane in hypothalamus from OVX control animals, a modest estrogen-induced increase in receptor at the plasma membrane without a corresponding increase in internalized receptor significantly decreased the percentage of receptor internalized ($p < 0.05$; Table 3). Estrogen shifted the ratio of receptor at the plasma membrane to receptor in light vesicles from about 5:1 in control to 10:1 in estrogen-treated hypothalamus. Estrogen treatment did not modify α_2-adrenoceptor density or localization in the POA (Table 3).

Discussion

Our findings suggest that estrogen acts over a relatively long period of time (between 24 and 48 h) to uncouple α_2-adrenoceptors from G proteins in the hypothalamus by stabilizing a phosphorylated state of the receptor. When agonist affinity for α_2-adrenoceptors was evaluated either by norepinephrine displacement of [^{3}H] RX 821002 or by binding of [^{3}H] UK 14304, we consistently found that in vivo exposure for 48 h to estrogen reduced high-affinity agonist binding. The total population of α_2-adrenoceptors in the hypothalamus, as reflected by the density of RX 821002 binding, significantly increased during the same 48-h exposure to estrogen *(5,6)* (Table 3). The reduced binding of the high-affinity agonist UK 14304 observed in membranes from rats exposed to estrogen for 48 h could be restored to control levels when hypothalamus slices were incubated with a PP2A activator for 30 min before membrane preparation (Table 1) or when hypothalamus membranes were incubated with exogenous alkaline phosphatase for 2 h (Table 2). These data are consistent with the hypothesis that estrogen uncouples α_2-adrenoceptors from G protein by inducing stable phosphorylation of the receptor. We use the term *stable phosphorylation* because α_2-adrenoceptor–G protein uncoupling is maintained following tissue homogenization, freezing, and thawing. Moreover, the ability of brief treatment of hypothalamus slices with the PP2A activator protamine and treatment of hypothalamus membranes with alkaline phosphatase to reinstate high-affinity agonist binding indicates that the uncoupled α_2-adrenoceptors can rapidly return to a G protein–coupled state once they are dephosphorylated.

Estrogen may stabilize receptor phosphorylation by reducing the rate of α_2-adrenoceptor dephosphorylation and resensitization in the hypothalamus. Treatment with estrogen for 2 d decreased the percentage of internalized α_2- (Table 3) and β-adrenoceptors *(7)* in the hypothalamus. During agonist-mediated desensitization, receptors are phosphorylated at the cell surface by GRK and uncoupled from G protein. The phosphorylated, uncoupled receptors then bind β-arrestin and internalize into endocytotic vesicles, which are associated with high levels of PP2A activity *(23)*. Following internalization, receptors are dephosphorylated and recycled back to the cell surface, allowing recoupling of receptor to G protein. Prolonged inhibition of receptor internalization can produce an irreversible desensitization of G protein–coupled receptor signaling *(14)*. We found that exposure to estradiol for 2 d decreased the number of high-affinity α_2-adrenoceptors in the hypothalamus and reduced the percentage of internalized α_2-adrenoceptors in the hypothalamus by approx 50% (Table 3). Furthermore, the same exposure to estrogen also decreased the percentage of internalized β-adrenoceptors and attenuated β-adrenergic stimulation of adenylyl cyclase in the hypothalamus *(7)*. These actions of estrogen are reversed by phosphatase activation. Thus, estrogen may inhibit α_2- and β-adrenoceptor resen-

sitization in the hypothalamus by reducing receptor internalization and dephosphorylation.

Estrogen inhibition of α_2-adrenoceptor resensitization in the hypothalamus may be causally related to reduced phosphatase activity. G protein–coupled receptors are phosphorylated by several serine/threonine kinases, leading to receptor–G protein uncoupling *(8,10)*. Following phosphorylation, G protein–coupled receptors must be dephosphorylated by phosphatases to regain the ability to couple to G protein *(16)*. Inhibition of phosphatase activity with okadaic acid increases α_{1B}-adrenoceptor phosphorylation and decreases receptor–G protein coupling in a cell expression system *(9)*. Moreover, altered function of the ryanodine receptor/calcium release channels from failing hearts correlates with increased receptor phosphorylation and decreased association of phosphatase 1 and PP2A with the receptor *(24)*. We demonstrate here that estrogen exposure significantly reduces phosphatase activity in the hypothalamus (Fig. 3). Therefore, estrogen-induced reductions in phosphatase activity in the hypothalamus may decrease the rate of α_2-adrenoceptor dephosphorylation and resensitization, stabilizing α_2-adrenoceptors in a phosphorylated, uncoupled state.

Our data suggest that estrogen regulates PP2A enzymatic activity in the hypothalamus. The major phosphatase believed to dephosphorylate and resensitize G protein–coupled receptors is a PP2A *(17,18)*. Our assay for phosphatase activity used protamine as a substrate and was carried out in the presence of manganese. Manganese activates both phosphatase 1 and PP2A, whereas protamine activates PP2A but inhibits phosphatase 1. Therefore, our phosphatase activity assay should have measured primarily PP2A activity. Furthermore, earlier studies *(25)* demonstrating estrogen attenuation of phosphatase activity in the rat uterus identified the estrogen-regulated activity as a PP2A. The ability of protamine to restore high-affinity α_2-adrenoceptor binding in hypothalamus membranes from estrogen-exposed rats suggests that suppression of PP2A activity is owing primarily to inhibition of the enzymatic activity rather than downregulation of the phosphatase.

A candidate mechanism for inhibition of phosphatase activity is regulation of endogenous phosphatase inhibitors. DARPP-32, a phosphatase 1 inhibitor that is highly expressed in brain, is activated by serine/threonine phosphorylation *(26)*. The steroid hormone progesterone facilitates phosphorylation of DARPP-32 in hypothalamus of estrogen-primed female mice *(27)*, thereby inhibiting phosphatase 1 activity. Thus, a similar mechanism could underlie estrogen-induced inhibition of PP2A in the hypothalamus.

Reductions in GRK activity in the hypothalamus following estrogen exposure may also contribute to the attenuation of receptor resensitization. GRK phosphorylation of receptors promotes binding of β-arrestin, which initiates receptor internalization *(11,28)*. GRK also cointernalizes with phosphorylated, uncoupled receptors *(29)*. Although the precise function of GRK internalization is not known, it has been hypothesized to play a role in receptor resensitization and sorting *(30)*. We show that estrogen reduces GRK mRNA, protein, and activity 24 h prior to any observable effects on α_2-adrenoceptor coupling to G protein in the hypothalamus. Thus, estrogen-induced suppression of GRK may reduce the rate of β-arrestin binding and receptor internalization, thereby decreasing receptor resensitization. Furthermore, reductions in GRK may inhibit receptor sorting to vesicles, where they are dephosphorylated and recycled back to the cell surface. Thus, estrogen-induced decreases in GRK mRNA, protein, and activity may attenuate receptor internalization and recycling.

Our findings also suggest potential mechanisms for estrogen-induced increases in total α_2-adrenoceptor density in the hypothalamus *(6)* (Table 3). Receptor internalization is normally followed by receptor resensitization and recycling back to the membrane or by degradation *(8)*. We find that treatment with estrogen inhibits α_2-adrenoceptor internalization. Decreasing α_2-adrenoceptor internalization might not only reduce receptor recycling but also receptor degradation. Over time, a reduced rate of receptor degradation with continued receptor synthesis could increase the total density of α_2-adrenoceptor binding sites. Alternatively, in response to reduced receptor coupling to G protein, neurons may elevate receptor synthesis to compensate for the decrease in receptor signaling.

Stabilizing α_2-adrenoceptor phosphorylation may be a specific example of a more general mechanism of estrogen-induced desensitization of G protein–coupled receptors. As noted previously *(4,6)*, estrogen decreases α_2-adrenergic receptor function and coupling to G protein in the hypothalamus. The same treatment with estrogen reduces β-adrenergic activation of adenylyl cyclase in the hypothalamus, apparently by decreasing β-adrenoceptor coupling to G protein *(31)*. Recent studies show that estrogen attenuates β-adrenoceptor function in the hypothalamus by stabilizing receptor phosphorylation and inhibiting receptor internalization *(7)*. Similarly, identical doses of estrogen attenuate the ability of μ-opioid receptors to inhibit the release of norepinephrine in the hypothalamus *(32)*. Behavioral effects of serotonin$_{1A}$ receptors in the hypothalamus are also attenuated after two weekly injections of high doses of estrogen *(33)*. Interestingly, activation of any of these receptors inhibits reproductive behavior and the release of gonadotropin. Therefore, the same mechanism hypothesized to underlie estrogen-induced decreases in α_2-adrenoceptor coupling to G protein may be responsible for the attenuation of multiple inhibitory inputs to the reproductive circuitry in the hypothalamus. The coordination of reproductive behavior and the release of gonadotropin in female rats may require estrogen not only to enhance excitatory inputs, but also to simultaneously attenuate inhibitory inputs to hypothalamus neurons.

Materials and Methods

Preparation of Animals

Female Sprague-Dawley rats weighing 150–175 g (Taconic Farms, Germantown, NY) were OVX bilaterally. After 5–7 d they were injected subcutaneously with vehicle (peanut oil) or 2 µg of EB (Steraloids, Wilton, NH) at various times prior to killing. Animals were decapitated, brains were removed, and the hypothalamus and POA were dissected as previously described *(22)*. In some experiments, 350-µm slices were made of the hypothalamus-POA block. The entire POA-hypothalamus was sliced on a McIlwain tissue chopper beginning approx 2 mm anterior to the optic chiasm and ending 1 mm anterior to the mammillary bodies. All animal experimentation was carried out in accordance with guidelines recommended by the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Preparation of Membranes

Total cell membranes were prepared by homogenization of dissected tissue in ice-cold lysis buffer (50 m*M* Tris; 10 m*M* $MgCl_2$; pH 7.4) for 30 s on ice. The homogenate was centrifuged for 10 min at 20,000*g*, the supernatant discarded, and the pellet frozen at −70°C. Before use pellets were resuspended in lysis buffer. To isolate vesicular membranes from plasma membranes, hypothalamus and POA tissue was fractionated on sucrose density gradients. The hypothalamus and POA from four animals were pooled by brain region and immediately placed into 2.5 mL of ice-cold lysis buffer. Pooled tissue was homogenized on ice for 20 strokes in glass-Teflon Dounce homogenizers and layered on top of discontinuous sucrose density gradients consisting of 3.5 mL of 55% (w/v), 3.5 mL of 32%, and 1.5 mL of 5% sucrose in lysis buffer. Gradients were centrifuged at 4°C for 60 min at 151,000*g* in a Beckman SW41 rotor. The 5/32% sucrose interface (light-vesicle fraction) and the 32/55% sucrose interface (plasma membrane fraction) were collected as described elsewhere *(13,34,35)*. Membrane fractions were immediately resuspended in lysis buffer and frozen at −70°C until use.

Phosphatase Treatments

Phosphatase activation was via either protamine stimulation of endogenous phosphatase activity in brain slices *(36,37)* or application of exogenous alkaline phosphatase to membrane preparations *(20,38,39)*. Hypothalamus and POA slices were equilibrated at 35°C in 300 µL of artificial cerebral spinal fluid for 75 min in an O_2/CO_2 saturated environment *(22)*. Slices were treated with vehicle (control) or 200 µg/mL of protamine (Sigma, St. Louis, MO), a PP2A activator, for 30 min. Total membranes were then prepared from slices as for dissected tissue. Alkaline phosphatase-treated membranes were prepared from hypothalamus and POA from control and EB-treated animals. Prior to ligand-binding assay, membranes were resuspended in 3 mL of lysis buffer and incubated with vehicle or 100 U/mL of alkaline phosphatase (Sigma) for 2 h at 37°C.

Radioligand-Binding Assays

To measure the affinity of α_2-adrenoceptors for the agonist norepinephrine in the hypothalamus and POA, the antagonist [^{3}H] RX821002 was displaced with increasing concentrations of unlabeled norepinephrine as described previously *(5)*. Parallel incubations were done in the presence of 100 µ*M* Gpp(NH)p to shift all α_2-adrenoceptors into the agonist low-affinity state. To measure the total density of α_2-adrenoceptors in different membrane fractions, [^{3}H] RX821002 binding was also assayed as previously described *(6)*. Aliquots of resuspended membranes were incubated in lysis buffer at 25°C for 4 h with six concentrations (0.2–10 n*M*) of [^{3}H] RX821002 in duplicate (light-vesicle fraction) or triplicate (plasma membrane fraction) in the absence or presence of 10 µ*M* nonradioactive RX821002 to define nonspecific binding.

To determine the effects of hormone treatment and phosphatase activation on the agonist high-affinity state of α_2-adrenoceptors, [^{3}H] UK 14304 (New England Nuclear, Boston, MA) binding was measured in total cell membranes. [^{3}H] UK 14304 binds selectively to the agonist high-affinity state of the α_2-adrenoceptor *(5)*, which represents receptors uncoupled from G protein. Membranes were incubated overnight at 0°C with six concentrations (0.1–15 n*M*) of [^{3}H] UK 14304 in duplicate in the absence or presence of 10 µ*M* nonradioactive RX821002 to define nonspecific binding.

To assess the density of Na^+/K^+-ATPase in light-vesicle and plasma membrane fractions, aliquots of resuspended membranes were incubated at 37°C for 2 h with a saturating concentration (2 µ*M*) of [^{3}H] ouabain (Amersham Life Sciences, Arlington Heights, IL). Binding reactions were done in duplicate in the absence or presence of 2 m*M* nonradioactive ouabain to define nonspecific binding.

All binding reactions were terminated by rapid filtration through glass fiber filters presoaked in 1% polyethyleneimine for 1 h. Protein concentrations in all experiments were determined by modified Lowry assay *(40)*. Norepinephrine displacement data were fit to sigmoidal curves using the curve-fitting program Radioligand (Biosoft). Data were converted to *pKi* using the Cheng-Prussoff equation (K_d obtained from RX821002 binding experiments on plasma membrane fractions) *(41)*. Biphasic fits were accepted only if $p < 0.05$. Saturation binding data were plotted and analyzed by the method of Scatchard using EBDA (Biosoft). The percentage of receptor in light-vesicle and plasma membrane fraction was calculated only when both fractions yielded an analyzable Scatchard plot.

Immunoblots

Protein was prepared by Dounce homogenization of dissected tissue in hot lysis buffer (1% sodium dodecyl sulfate [SDS]; 1 m*M* sodium vanadate; 0.1 m*M* phenylmethylsul-

fonyl fluoride (PMSF), and 10 m*M* Tris-HCl, pH 7.4). Following homogenization, tissue lysate was microwaved for 10–15 s, boiled for 10 min, and centrifuged for 5 min at 10,000*g* to remove insoluble material. Duplicate samples (25 μg of protein) were subjected to electrophoresis on 12.5% SDS-polyacrylamide minigels, transferred electrophorectically to nitrocellulose, blocked for 30 min with 5% nonfat dry milk in Tris-buffered saline (TBS), and incubated overnight at room temperature with primary antibody. Primary antibodies were GRK 2 rabbit polyclonal antibody (1:500) (Santa Cruz Biotechnologies, Santa Cruz, CA), β-tubulin type I and II mouse monoclonal antibody (mAb) (1:2000) (Sigma), SNAP-25 mouse mAb (1:20), and carboxypeptidase E, rabbit polyclonal antibody (1:1000). GRK 2 blots were washed three times with 1X TBS for 1 min, twice with 1X TBS and 0.1% Tween-20 for 15 min, and then twice with 1X TBS for 1 min. Membranes were blotted for β-tubulin following initial blotting for the GRK 2 isoform to verify that protein load was comparable among samples. SNAP-25, carboxypeptidase E, and β-tubulin blots were washed five times with 1X TBS for 1 min. Control blots were incubated with normal rabbit serum. All blots were incubated at room temperature for 3 h with horseradish peroxidase–conjugated antirabbit or antimouse secondary antibodies (1:1000) (Boehringer Mannheim, Indianapolis, IN) when appropriate. Blots were then washed as for the primary antibody, and peroxidase activity was visualized by chemiluminescence according to the manufacturer's instructions (Amersham Life Sciences).

Blots were exposed to film for two different time periods to obtain signals within the linear range of the film. Data were expressed as optical density (OD) of the GRK 2 isoform signal corrected for variability in protein load by the OD of the β-tubulin signal. ODs were obtained by photographing autoradiograms using a Kodak Digital Science DC 120 camera and analyzing the image using Kodak Digital Science Analysis Software (Eastman Kodak, Rochester, NY).

GRK Activity Assay

GRK activity was assayed using a modified version of published methods *(42–45)*. Hypothalamus and POA were dissected, rinsed in phosphate-buffered saline, and homogenized in a Dounce homogenizer in ice-cold lysis buffer (25 m*M* Tris, pH 7.5; 5 m*M* EDTA; 5 m*M* EGTA; 1 m*M* PMSF; 10 μg/mL of leupeptin). Homogenates were centrifuged for 30 min at 47,000*g* at 4°C; the supernatant was removed and labeled cytosol. The pellet was resuspended in lysis buffer containing 250 m*M* NaCl and left on ice for 30 min to extract membrane-associated proteins. The suspension was then centrifuged for 30 min at 47,000*g* at 4°C, and the supernatant was removed and labeled membrane. Triton X-100 (0.02%) was added to stabilize the enzyme *(44)* and samples were stored at 4°C until use.

GRK activity was determined by incubating cytosol and membrane fractions with a synthetic peptide substrate (RREEEEESAAA) that is phosphorylated by GRK 2 and GRK 3. Samples were assayed in triplicate for 60 min at 30°C in a mixture (final volume of 25 μL) containing approx 10 μg of protein, 1 m*M* synthetic peptide, 100 μ*M* [γ-^{32}P] adenosine triphosphate (100–500 cpm/pmol) (Amersham Life Sciences), 2 m*M* EDTA, 7.5 m*M* $MgCl_2$, 25 μ*M* KN-62 (a calcium calmodulin kinase inhibitor), 20 m*M* Tris-HCl (pH 7.5), and 40 μg/mL of phosphatidylserine. Nonspecific activity was determined in duplicate samples with the addition of 125 U of heparin, a GRK inhibitor. Following enzyme activation and incubation, 20 μL of the reaction mixture was spotted onto P-81 phosphocellulose paper, which was washed in 1% phosphoric acid and counted in a scintillation counter. The specific activity of GRK was determined by subtracting activity in the presence of inhibitor from total activity. This method may overestimate actual GRK activity if casein kinase II, which phosphorylates the substrate peptide and is inhibited by heparin *(46)*, is present in the extract. However, casein kinase II is not regulated by estrogen in other systems *(47)*. All assays were performed under conditions in which activity was linear with respect to both protein and time (data not shown).

Northern Blots for GRK 2

Total RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH). RNA purity and concentration were determined by ultraviolet (UV) spectrophotometry. Total RNA (15 μg) was electrophoresed on 1.2% agarose gels, transferred to positively charged nylon membrane Hybond-N+ (Amersham Life Sciences), and immobilized by exposing membranes to UV light for 5 min and baking for 4 h at 80°C. Probes were made by labeling DNA fragments of GRK 2 and β-actin cDNAs with [α^{32}P]-dCTP random priming (Promega, Madison, WI). Hybridization was overnight at 48°C with 2 to 3×10^7 cpm/mL of probe in 5 mL of hybridization buffer (0.5% SDS, 50% formamide, 10% dextran sulfate, 5X saline sodium citrate [SSC] buffer, 0.125 mg/mL of salmon sperm DNA). Membranes were washed for 20 min twice with 2X SSC at room temperature, once in 1X SSC at room temperature, once in 1X SSC at 55°C, twice in 0.5X SSC at 55°C, once in 0.1X SSC at 55°C, and once in 0.1X SSC at 60°C. Membranes were then exposed to Kodak X-AR film at –70°C for 4–7 d. Membranes were initially probed for GRK 2 mRNA, then for β-actin mRNA to verify that RNA load was comparable among samples. Data were expressed as OD of the GRK 2 mRNA signal corrected for variability in RNA load by the OD of the β-actin signal. ODs were obtained after photographing autoradiograms as described for immunoblots.

Phosphatase Activity Assay

Hypothalamus and POA were dissected and homogenized in a Dounce homogenizer in ice-cold lysis buffer (20 m*M* Tris-HCl, pH 7.5; 1 m*M* EDTA; 1 m*M* EGTA; 50 m*M*

β-mercaptoethanol, 1 m*M* PMSF, 0.25 *M* sucrose). Homogenates were centrifuged for 10 min at 1000*g* at 4°C; the supernatant was removed and centrifuged for 60 min at 47,000*g* at 4°C. The supernatant (cytosol) was stored at −70°C until use. To measure phosphatase activity, the amount of ^{32}P released from PKC-phosphorylated protamine was measured according to a published protocol *(25)*. Briefly, cytosol was incubated at 25°C with ^{32}P-labeled protamine sulfate (4 mg/mL) in a reaction mixture containing 20 m*M* Tris, 30 m*M* β-mercaptoethanol, 1.25 mg/mL of bovine serum albumin, and 29 m*M* $MnCl_2$ (phosphatase activator; *[48]*). Nonspecific activity was determined by incubating without cytosol or with heat-inactivated brain cytosol (100°C for 10 min). All assays were performed under conditions in which activity was linear with respect to both protein and time (data not shown).

Statistical Analyses

GRK immunoblot data were analyzed using repeated measures analysis of variance (ANOVA). Samples in duplicate were run on gels with each hormonal condition represented. Samples run on the same gel were considered related. Post hoc analysis used paired *t*-tests. GRK activity and GRK 2 Northern data were analyzed using one-way ANOVA. Significant differences between means were determined for main effects by Fisher's test. [^3H] UK 14304 binding data were analyzed using two-way ANOVA with hormone and drug treatment as factors. Significant differences between means were determined for main effects by Fisher's test. Other binding data and phosphatase activity data were analyzed using student's *t*-tests. In all cases, significance was defined as $p < 0.05$.

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