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Functional Interactions between Estrogen and Insulin-Like Growth Factor-I in the Regulation of α_{1B}-Adrenoceptors and Female Reproductive Function

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The ovarian hormone estradiol (E_2) and insulin-like growth factor-I (IGF-I) interact in the CNS to regulate neuroendocrine function and synaptic remodeling. Previously, our laboratory showed that 2 d E_2 treatment induces α_{1B}-adrenoceptor expression and promotes IGF-I enhancement of α_1-adrenoceptor potentiation of cAMP accumulation in the preoptic area (POA) and hypothalamus (HYP). This study examined the hypothesis that E_2-dependent aspects of female reproductive function, including α_{1B}-adrenoceptor expression and function in the POA and HYP, are mediated by brain IGF-I receptors (IGF-IRs) in female rats. Ovariohysterectomized rats were implanted with a guide cannula aimed at the third ventricle and treated *in vivo* with vehicle or E_2 daily for 2 d before experimentation. Intracerebroventricular infusions of JB-1, a selective IGF-IR antagonist, were administered every 12 hr beginning 1 hr before the first E_2 injection. Administration of JB-1 during E_2 priming completely blocks hormone-induced luteinizing hormone release and partially inhibits hormone-dependent reproductive behavior. Reproductive behavior is restored by intracerebroventricular infusion of 8-bromo-cGMP, the second messenger implicated in α_1-adrenergic facilitation of lordosis. In addition, blockade of IGF-IRs during E_2 priming prevents E_2-induced increases in α_{1B}-adenoceptor binding density and abolishes acute IGF-I enhancement of NE-stimulated cAMP accumulation in HYP and POA slices. These data document the existence of a novel mechanism by which IGF-I participates in the remodeling of noradrenergic receptor signaling in the HYP and POA after E_2 treatment. These events may help coordinate the timing of ovulation with the expression of sexual receptivity.

Key words: estradiol; IGF-I; adrenoceptor; hypothalamus; preoptic area; reproduction

Estradiol (E_2), the main estrogenic hormone produced and secreted by the ovaries, acts on the hypothalamus (HYP) and preoptic area (POA), brain regions responsible for female reproductive function (Barfield and Chen, 1977; Chappel, 1985). E_2 actions in these brain regions enhance reproductive success by ensuring that female sexual receptivity (lordosis) coincides with the release of pituitary luteinizing hormone (LH), which triggers ovulation (Pfaff, 1980; Etgen et al., 1992; Freeman, 1994).

E_2 action in the HYP and POA modifies several cellular and molecular components of the noradrenergic system. Norepinephrine (NE) has been implicated as a major neurochemical mediator of both sexual receptivity and the preovulatory LH surge (Kalra and Kalra, 1983; Crowley, 1986; Etgen et al., 1992; Freeman, 1994; Herbison, 1997). NE actions in the HYP–POA can either facilitate or inhibit lordosis behavior and LH release, depending on the hormonal status of the animal (Etgen et al., 1992). NE activation of α_1-adrenoceptors facilitates lordosis behavior and LH release only if animals have been exposed to E_2 (Crowley, 1986; Kow et al., 1992; Weesner et al., 1993; Herbison, 1997). E_2 increases α_{1B}-adrenoceptor binding and mRNA levels in both HYP and POA (Petitti et al., 1992; Karkanias et al., 1996). These NE receptors are believed to mediate NE facilitation of sexual receptivity and preovulatory LH release (Kow et al., 1992; Hosny and Jennes, 1998). Despite evidence that E_2 remodels the biochemical responses of the HYP and POA to NE, the mechanism or mechanisms by which E_2 modulates the noradrenergic system are still unclear.

Cross-talk or interactions between E_2 and insulin-like growth factor-I (IGF-I) have been demonstrated in the CNS, in various reproductive tissues and in cell cultures. For example, E_2 can regulate the expression of IGF-I, IGF-I binding proteins, and IGF-I receptors (IGF-IRs) (Dickson et al., 1986; Pons and Torres-Aleman, 1993; Wimalasena et al., 1993; Sahlin et al., 1994). Likewise, IGF-I can regulate the expression and function of E_2 receptors (Aronica and Katzenellenbogen, 1993; Stoica et al., 2000). In addition, IGF-I has been implicated in certain effects of E_2 on synaptic structure and on neuroprotection (Duenas et al., 1996; Garcia-Segura et al., 1996; Patrone et al., 1996; Azcoitia et al., 1999; Fernandez-Galaz et al., 1999). Recently, we showed that IGF-I enhances α_1-adrenoceptor function in the HYP and POA, but only in E_2-primed rats (Quesada and Etgen, 2001). Because these observations suggest that E_2 effects on NE receptor function may involve IGF-I, we hypothesized that E_2-dependent sexual receptivity, LH release and remodeling of the biochemical responses of HYP and POA slices to NE may be mediated via IGF-IR activity. Present data show that *in vivo* blockade of brain IGF-IRs during E_2 priming prevents increased expression of α_{1B}-adrenoceptor in the HYP–POA and completely blocks E_2-dependent LH release. *In vivo* blockade of IGF-IRs during E_2 priming also blocks IGF-I enhancement of α_1-adrenoceptor sig-

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naling in POA and HYP slices and partially inhibits E_2-dependent sexual receptivity. These novel findings demonstrate that brain IGF-IRs are involved in the cellular and molecular actions underlying E_2 regulation of female reproductive function.

MATERIALS AND METHODS

Animal treatments. Female Sprague Dawley rats (Taconic Farms, Germantown, NY) weighing 175–200 gm were anesthetized with ketamine (80 mg/kg body weight) and xylazine (4 mg/kg body weight), placed into a stereotaxic apparatus, and secured with ear bars and a nosepiece set at +5.0 mm. A 26 gauge guide cannula (Plastics One, Roanoke, VA) was implanted into the third ventricle using coordinates from the atlas of Pellegrino et al. (1979). Animals were bilaterally ovariohysterectomized (OVX) to remove the primary source of estrogen and progesterone immediately after stereotaxic surgery. Four to seven days after stereotaxic surgery and OVX, all rats were injected subcutaneously with either peanut oil (control) or with 2 μg of E_2 benzoate (EB) (Steraloids Inc., Wilton, NH) in peanut oil 24 and 48 hr before killing. In most cases, multiple infusions of 1 μl of saline or 4 μg of JB-1 (Bachem, San Carlos, CA), a selective antagonist for IGF-IR, in 1 μl of saline were given intracerebroventricularly 1 hr before and 12 hr after both EB injections. Experimental use of animals was in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine approved all animal protocols.

NE-stimulated cAMP accumulation. The animals were rapidly killed, and the brains were placed in artificial CSF (aCSF) (Yamamoto, 1972). In all experiments, brain slices were prepared between 1000 and 1100 hr to eliminate potential diurnal variation in cAMP (Kant et al., 1981) and adrenoceptor (Krauchi et al., 1984; Jhanwar-Uniyal et al., 1986) content. HYP and POA were dissected, and 350 μm slices were made on a McIlwain tissue chopper beginning ~2 mm anterior to the optic chiasm and ending 1 mm anterior to the mammillary bodies. The first four slices containing the medial and lateral POA, suprachiasmatic nucleus, and supraoptic nucleus were taken and designated POA. The next slice was discarded, and the following four slices containing the anterior, lateral, ventromedial, paraventricular, arcuate, and dorsomedial nuclei of the HYP were kept and designated HYP. Individual slices were incubated at 35°C in 300 μl of oxygenated aCSF, which included a phosphodiesterase (PDE) inhibitor, 1 mM 3-isobutyl-1-methylxanthine (IBMX). IBMX was dissolved in absolute ethanol and added to slices such that the final concentration of ethanol was 1%. After a 75 min equilibration period slices were exposed for 15 min to 10 nM IGF-I (National Institutes of Health, National Hormone and Pituitary Program) dissolved in distilled water. Slices were then stimulated with either vehicle or 100 μM NE (Research Biochemicals, Natick, MA) dissolved in 0.01 N HCl. Each experiment included POA and HYP slices from EB-treated animals infused with either saline or JB-1, and each experiment was repeated four times. cAMP determination was done as previously described (Quesada and Etgen, 2001) using a modified Gilman cAMP assay (Brostrom and Kon, 1974).

Immunoblotting for phosphorylated extracellular receptor-activated kinase 1/2. HYP slices from E_2-primed rats that were also infused with JB-1 or saline were incubated at 35°C in 300 μl of oxygenated aCSF for 75 min, then exposed for 15 min to vehicle or 10 nM IGF-I dissolved in distilled water. After vehicle or IGF-I treatments, two slices were pooled and prepared by Teflon homogenization in ice-cold 1% Triton X-100, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 mM Tris-HCl, pH 7.4. After homogenization, samples were spun for 15 min at 14,000 × *g* to remove insoluble material. Protein concentrations were determined by a modified Lowry assay. Fifty micrograms of protein were applied to 12.5% SDS-polyacrylamide minigels and resolved at 150 V for 1.5 hr. Proteins were transferred electrophoretically onto polyvinylidene-diflouride membranes (Renaissance; New England Nuclear, Boston, MA) at 150 amps for 1 hr. Membranes were then blocked for 1 hr in 5% bovine albumin serum (BSA) and 0.1% Tween 20 in Tris-buffered saline (TBS) at 37°C. Membranes were incubated for 1 hr at 37°C with an antibody for phosphorylated extracellular receptor-activated kinase 1/2 (ERK1/2) (Tyr 204; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Blots were stripped by incubating the membranes for 30 min at 50°C in stripping solution (0.0625 M Tris-HCl, pH 6.8; 0.2% SDS, 0.1 M β-mercaptoethanol), washed three or four times in TBS for 10 min, and reprobed with rabbit anti-ERK2 (1:1000; Santa Cruz Biotechnology) for total ERK2 protein assessment. Binding of the primary antibody was detected by anti-rabbit secondary antibodies conjugated to horseradish peroxidase (1:10,000) in 5% BSA. Peroxidase activity was visualized by means of chemiluminescence. Blots were exposed to FUJI medical x-ray film (Fisher Scientific, Pittsburgh, PA). Band intensity was obtained by scanning autoradiograms on a DC-120 digital camera (Eastman Kodak, Rochester, NY) with a +3 diopter lens and analyzing the image using the Kodak 1D gel analysis program. Immunoblots were quantitatively analyzed by taking the ratio of the optical density (OD) of the phospho-ERK2 band to the OD of the total ERK2 band. That ratio was used to calculate the percentage of change in ERK2 activation of IGF-I versus vehicle-treated HYP slices run on the same gel.

Radioligand binding assays. To provide sufficient material for Scatchard analysis, tissue from two rats given identical hormone injections and drug treatments was combined. POA and HYP samples were homogenized separately in 5 ml of ice-cold Tris-$MgCl_2$ buffer (50 mM Tris HCl and 10 mM $MgCl_2$, pH 7.4) using a Polytron at speed 5–6 for 20 sec. The homogenates were centrifuged for 10 min at 20,000 × *g*, the supernatant was discarded, and the pellet containing the membrane fraction was frozen at −70°C until assay. Freezing of the crude membrane fraction does not result in a measurable loss of any NE receptor subtype (Etgen and Karkanias, 1990).

The radioligand ^{3}H-prazosin (87 Ci/mmol; New England Nuclear) was used to measure total α_1-adrenoceptor binding in brain membranes. To distinguish α_{1A} and α_{1B}-adrenoceptor subtypes, chlorethylclonidine (CEC; Research Biochemicals), a selective, irreversible inactivator of the α_{1B}-adrenoceptor, was used. Frozen membranes were resuspended in 6 ml of Na-HEPES buffer and split into two equal fractions. Each fraction was preincubated for 10 min at 37°C with vehicle or with 10 μM CEC. Reactions were stopped by addition of 6 ml of ice-cold Na-HEPES buffer and centrifugation for 10 min at 20,000 × *g*. The supernatant was discarded, and the pellet was resuspended in 6 ml of Tris-$MgCl_2$ buffer. For Scatchard analysis, duplicate 200 μl aliquots were incubated for 20 min at 37°C with 0.05–5 nM ^{3}H-prazosin with and without a 2000-fold excess of phentolamine to assess nonspecific binding. Specific ^{3}H-prazosin binding after CEC inactivation reflects the α_{1A}-adrenergic receptor population. The α_{1B}-adrenoceptor population was determined by subtracting the binding of ^{3}H-prazosin after CEC inactivation from total specific ^{3}H-prazosin binding. Bound and free ^{3}H-prazosin were separated by rapid filtration through glass fiber filters (FPB-148 Whatman GF/B) on a Brandel (Gaithersburg, MD) cell harvester as described previously (Petitti et al., 1992). Ligand affinities (K_d), apparent receptor numbers (B_{max}), and Hill coefficients were calculated using the EBDA ligand program (Elsevier-Biosoft, Cambridge, UK). Experiments used tissue from OVX control and EB-primed female rats infused chronically with either saline or JB-1 and were repeated four times.

LH radioimmunoassay. Concentration of LH in serum was determined using primary antibody for rat LH (1:30,000; National Hormone and Pituitary Program) and ^{125}I-LH (3000–5000 cpm/100 μl; Covance Laboratories, Inc., Vienna, VA). Secondary antibody, goat anti-rabbit IgG, was obtained from Sigma (St. Louis, MO). Concentration of rat LH was expressed as ng RP-3 per ml, provided by the National Hormone and Pituitary Program. Animals were maintained on a reverse 14:10 light/dark cycle with the lights off at 11:00 A.M. Trunk blood samples were collected when the animals were decapitated at 8:00 P.M., allowed to clot overnight at 4°C, then centrifuged at 2000 × *g* for 30 min. Serum was decanted into polypropylene tubes and frozen at −20°C until analysis. Concentrations of LH in 100 μl aliquots of serum were determined in triplicate. Incubation periods between additions of primary antibody, radioiodinated hormone, and second antibody were 24 hr at room temperature. The sensitivity of the assay was 0.05 ng/tube, and samples were run in two separate assays. The intra-assay and inter-assay variances were 6 and 18%, respectively.

Reproductive behavior testing. Animals were maintained on a reverse 14:10 light/dark cycle with the lights off at 11:00 A.M. OVX female rats were primed with EB for 48 hr as described previously followed by 500 μg of progesterone 4 hr before behavior testing. Animals were tested once a week for 3 weeks after receiving one of three treatments in random order: (1) multiple infusions of JB-1 (4 μg/1 μl) dissolved in saline, (2) multiple infusions of 1 μl of saline, or (3) multiple infusions of JB-1 (4 μg/1 μl) followed by a single infusion of 8-bromo-cGMP (1 μg/2 μl) (Calbiochem, La Jolla, CA) administered 4 hr before behavior testing. Separate groups of rats received a single acute infusion of JB-1 (4 μg/1 μl) given either at 12 or 4 hr before testing (12 hr, $n = 6$; 4 hr, $n = 4$). Experienced stimulus male rats were placed in 20 gallon glass arenas and allowed to adapt for 10 min. Females were then placed in the

arenas with a male until they received 10 mounts with pelvic thrusting. A lordosis quotient (LQ = number of lordosis responses/number of mounts × 100) was used as a measure of behavioral receptivity. The quality of lordosis was also scored on a scale of 0–3 (0, no lordosis; 1, shallow lordosis; 2, definitive dorsiflexion of the spine; 3, exaggerated lordosis). Anatomical verification of the cannula placement was made according to the atlas of Pellegrino et al. (1979).

Statistics. cAMP levels were analyzed with one-way ANOVA using the Statview statistical program with drug treatment as the only factor. Significant differences between means were determined for main effects by Fisher's PLSD. Radioligand binding and LH data were analyzed using two-way ANOVA with drug treatment and hormone as the two factors. Significant differences between means were determined for main effects by Fisher's PLSD. Behavioral data were analyzed using repeated measures one-way ANOVA with drug as the repeated factor, followed by Fisher's PLSD. Differences were considered significant if $p < 0.05$.

RESULTS

E_2 induction of α_{1B}-adrenoceptor in the POA and HYP requires IGF-IR activity

Receptor tyrosine kinases such as IGF-I and insulin can increase α_{1D}-adrenoceptor gene expression and function in vascular smooth muscle cells (Hu et al., 1996), and general tyrosine kinase inhibitors can block NE upregulation of α_{1B}-adrenoceptors in clonal H cells (Bird et al., 1997). We examined the possibility that the E_2-dependent increase in α_{1B}-adrenoceptor density in the POA and HYP in female rats may require IGF-I activity. E_2-treated female rats received multiple intracerebroventricular infusions of JB-1 or saline, and receptor density for total α_1-adrenoceptor, α_{1A}- and α_{1B}-adrenoceptor was examined in HYP and POA membranes. JB-1 is a peptide analog of IGF-I based on the amino acid sequence of the C-terminal, D domain of IGF-I, which is involved in binding to the IGF-IR. It is a potent, highly selective, competitive antagonist of IGF-I-dependent receptor autophosphorylation and cellular proliferation (Pietrzkowski et al., 1992).

As reported previously, E_2 modestly increases the density of ^{3}H-prazosin binding sites (total α_1-adrenoceptor) in POA and HYP membranes from saline-infused animals ($p < 0.05$). Multiple intracerebroventricular infusions of JB-1 block E_2-induced increases in total α_1-adrenoceptor binding in POA and HYP (Fig. 1*A*). When the α_{1A}- and α_{1B}-adrenoceptor subtypes were pharmacologically distinguished, POA and HYP membranes from saline-infused, E_2-treated females demonstrate a fourfold to fivefold fold increase in α_{1B}-adrenoceptor density when compared with OVX control animals ($p < 0.05$). The density of α_{1A}-adrenoceptors is not affected by E_2 treatment (Fig. 1*B*). Multiple intracerebroventricular infusions of JB-1 prevent E_2 induction of α_{1B}-adrenoceptor expression in POA and HYP (Fig. 1*C*). Neither E_2 treatment nor infusions of JB-1 affect the binding affinity of ^{3}H-prazosin in POA and HYP membranes (Table 1).

E_2-induced LH surge is dependent on IGF-IR activity

Administration of E_2 to OVX female rats causes a daily LH surge (Caligaris et al., 1971). IGF-I can also affect LH release by altering gonadotropin-releasing hormone (GnRH) release from the HYP (Hiney et al., 1991; Soldani et al., 1994; Wilson, 1995; Hiney et al., 1996). We tested the hypothesis that steroid-induced LH release requires IGF-IR activation. OVX rats were given one of three hormone treatments (Oil, EB, or EB + progesterone; $N = 4–8$) and were infused every 12 hr with either JB-1 or saline. The estrogen priming consisted of two injections of EB (2 μg) 24 and 48 hr before killing. Progesterone (500 μg) was injected 44 hr after the first EB injection and 4 hr before killing. LH levels in OVX rats are chronically elevated because of the loss of steroid negative feedback (Ortmann et al., 1988). Multiple intracerebroventricular infusions of JB-1 have no effect on these elevated LH levels in OVX rats given no hormone replacement (Fig. 2). Treatment of OVX, saline-infused rats with E_2 alone tends to lower LH levels, but this effect is not statistically significant.

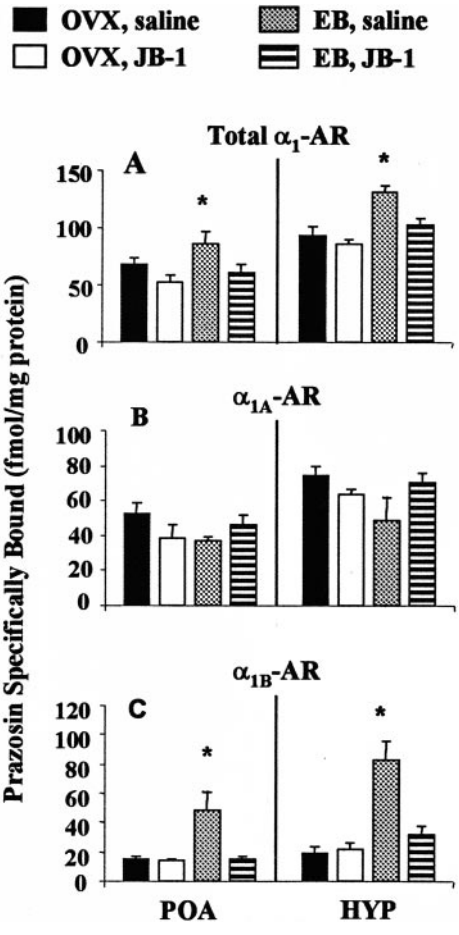

Figure 1. Effect of JB-1 infused into the third ventricle on E_2-induced α_{1B}-adrenoceptor (*AR*) density in the POA and HYP. Membranes from POA and HYP were prepared from OVX control and E_2-treated (*EB*) female rats infused with saline or JB-1 as described in Materials and Methods. *A,* Total ^{3}H-prazosin binding corrected for nonspecific binding reflects both α_1-AR subtypes. *B,* The α_{1A}-AR population is measured after CEC inactivation. *C,* The α_{1B}-AR population is determined by subtracting the binding of ^{3}H-prazosin after CEC inactivation from total ^{3}H-prazosin binding. B_{max} values were obtained by Scatchard analysis. The data presented are the means ± SEM from four independent replications. $^*p < 0.05$.

Table 1. Effects of JB-1 on ^{3}H-prazosin binding affinity

	K_D (nM ± SEM)			
	OVX		EB	
	Saline	JB-1	Saline	JB-1
POA	0.45 ± 0.08	0.70 ± 0.11	0.66 ± 0.11	0.82 ± 0.17
HYP	0.48 ± 0.10	0.54 ± 0.10	0.78 ± 0.15	0.64 ± 0.10

OVX female rats were primed with vehicle (OVX), or with EB (2 μg, 24 and 48 hr before killing). Multiple infusions of saline or JB-1 were given 1 hr before the first EB or vehicle injection and every 12 hr thereafter. K_D values were obtained from Scatchard analysis of ^{3}H-prazosin binding. Each value represents the mean of four independent replications. B_{max} values are shown in Figure 1*A*.

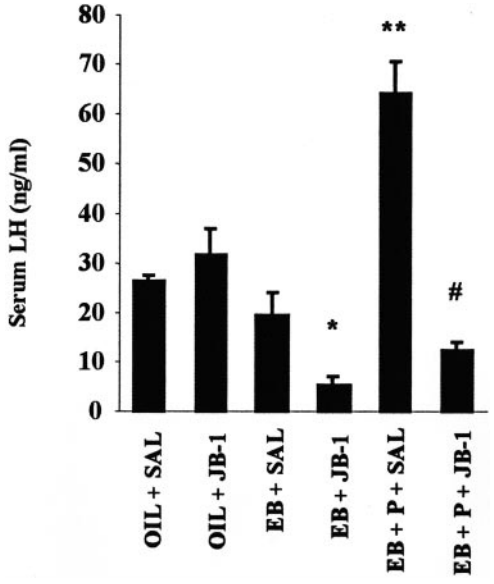

Figure 2. Effect of JB-1 infused into the third ventricle on hormone-induced LH secretion. Serum for LH radioimmunoassay was prepared as described in Materials and Methods. OVX rats were given one of the following treatments: OIL, EB, or EB + progesterone (*P*). Multiple intracerebroventricular infusions of vehicle saline (*SAL*) or JB-1 were given 1 hr before the first EB injection and every 12 hr thereafter. The data presented are the means ± SEM from four to eight independent replications. *OIL + SAL, OIL + JB-1, EB + SAL, and EB + P + SAL ($p < 0.05$); **all other groups ($p < 0.05$), #OIL + SAL, OIL + JB-1, and EB + P + SAL ($p < 0.05$).

However, LH levels in E_2-treated rats given multiple intracerebroventricular infusions of JB-1 are significantly lower than in OVX controls ($p < 0.05$). Because this result does not conclusively indicate if blockade of IGF-IR activity enhances E_2 negative feedback or inhibits E_2 positive feedback on LH secretion, we treated OVX rats with both E_2 and progesterone. LH release is further enhanced when E_2-treated, OVX female rats are subsequently given progesterone (Caligaris et al., 1971). The administration of E_2 plus progesterone significantly increases LH levels when compared with OVX controls ($p < 0.05$). Intracerebroventricular infusions of JB-1 during estrogen priming abolish the LH surge produced by administration of EB plus progesterone to OVX female rats ($p < 0.05$) (Fig. 2).

E_2-dependent sexual behavior is partially dependent on IGF-IR activity

To examine the possibility that E_2-dependent sexual behavior requires IGF-IR activity, E_2 plus progesterone-treated, OVX female rats received multiple intracerebroventricular infusions of JB-1 during estrogen priming. The E_2 priming dosages used in our experimental paradigm are subthreshold for lordosis behavior; administration of progesterone is required to facilitate sexual receptivity in these animals (Pfaff et al., 1994). JB-1 modestly but significantly ($p < 0.05$) decreases lordosis behavior (Fig. 3*A,B*). Recently, acute intracerebroventricular infusion of IGF-I was shown to facilitate female sexual behavior independent of E_2 priming (Apostolakis et al., 2000). Therefore, we assessed whether acute JB-1 infusion to block IGF-IRs near the time of lordosis testing would inhibit the behavior. Acute intracerebroventricular infusion of JB-1 at either 12 hr (Fig. 3*C*) or 4 hr (LQ: saline = 70 ± 4.0; JB-1 = 75 ± 2.9) before testing has no effect on lordosis behavior.

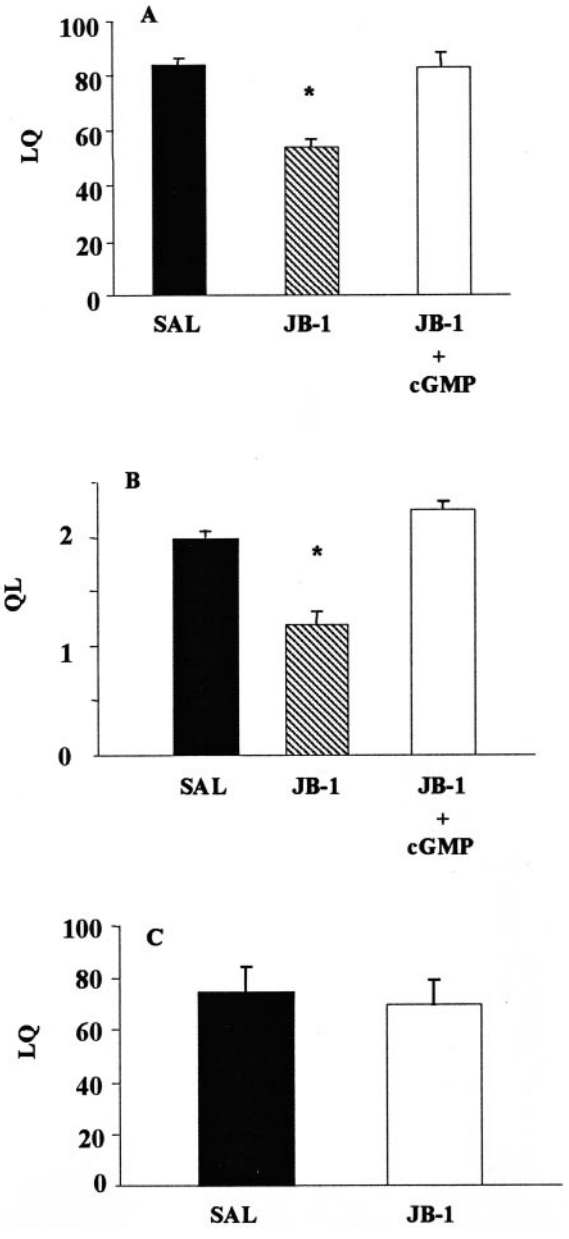

Figure 3. Effect of JB-1 infused into the third ventricle on lordosis behavior of E_2- and progesterone-primed female rats. *A,* Lordosis quotient (*LQ*) after chronic infusions of saline (*SAL*) or JB-1 alone or JB-1 followed by intracerebroventricular infusion of 1 μg of 8-bromo-cGMP 4 hr before behavior testing. *B,* Quality of lordosis (*QL*) in same animals shown in *A*. Values presented are the means ± SEM ($n = 9$). *$p < 0.05$. *C,* LQ after acute infusion of 4 μg of JB-1 or SAL 12 hr before behavior testing. Values presented are the means ± SEM ($n = 6$).

A likely mechanism by which α_1-adrenoceptors facilitate female sexual behavior is by stimulating cGMP synthesis, which in turn activates protein kinase G (Chu and Etgen, 1999). For example, intracerebroventricular infusion of 8-bromo-cGMP 4 hr before lordosis behavior testing reversed α_1-adrenoceptor antagonist inhibition of lordosis behavior (Chu and Etgen, 1999). Thus, we examined whether the inhibitory effects of multiple intracerebroventricular infusions of JB-1 on sexual behavior can be rescued by intracerebroventricular infusion of 8-bromo-cGMP. During one of their three weekly tests, E_2-primed animals receiving multiple intracerebroventricular infusions of JB-1 were infused

with 1 μg of 8-bromo-cGMP at the time of progesterone injection. Intracerebroventricular infusion of 8-bromo-cGMP reverses the inhibitory effects of multiple intracerebroventricular infusions of JB-1 on sexual behavior ($p < 0.05$) (Fig. 3*A*,*B*).

Blockade of IGF-IR during E_2 priming prevents E_2-dependent, IGF-I enhancement of NE-stimulated cAMP accumulation

We previously demonstrated that acute application of IGF-I enhanced NE-stimulated cAMP accumulation in POA and HYP slices, via modulation of α_1-adrenoceptor function, only if the animal is E_2-primed (Quesada and Etgen, 2001). The present study showed that blockade of IGF-IR during estrogen priming prevents E_2-dependent increases in α_{1B}-adrenoceptor binding in the HYP and POA. We therefore examined the hypothesis that E_2-dependent, IGF-I modulation of NE signaling in the POA and HYP of female rats requires IGF-IR activation during E_2 priming.

E_2-treated, OVX female rats received multiple intracerebroventricular infusions of JB-1 or saline every 12 hr during the 2 d E_2 priming period. IGF-I enhances NE-stimulated cAMP accumulation in POA and HYP slices of E_2-treated female rats infused with saline. Multiple intracerebroventricular infusions of JB-1 abolish IGF-I enhancement of NE-stimulated cAMP accumulation in POA and HYP slices of E_2-primed animals (Fig. 4). It is possible that residual JB-1 from the multiple intracerebroventricular infusions was present in the slices and blocked IGF-I potentiation of NE-stimulated cAMP accumulation. To determine if IGF-IRs could be activated by application of IGF-I onto brain slices, phosphorylation of ERK1/2 in response to acute application of IGF-I onto slices from JB-1 infused rats was monitored. The mitogen-activated protein kinase cascade, including ERK1 and ERK2, are known downstream substrates of IGF-IR activation (Zumkeller and Schwab, 1999). Figure 5 shows that multiple infusions of JB-1 do not interfere with the ability of acute IGF-I application to activate IGF-IRs, as measured by IGF-I-induced phosphorylation of ERK1/2 in HYP slices from E_2-treated rats.

DISCUSSION

The data presented herein demonstrate that the positive feedback effects of E_2 on LH release require IGF-IR activity in the brain and that E_2 priming of sexual receptivity also involves IGF-I action. Present results also show that E_2-induced increases in α_1-adrenoceptor binding and function in the HYP and POA require IGF-IR activity. Thus, our findings provide the first evidence that IGF-IRs in the brain are physiological mediators of multiple aspects of estrogen action on the hypothalamic–pituitary–gonadal axis and sexual behavior.

E_2 and IGF-I regulation of gene transcription

E_2 actions in the POA and HYP facilitate reproductive success by ensuring that the period of female sexual receptivity coincides with the release of LH, which triggers ovulation (Pfaff, 1980; Etgen et al., 1992; Freeman, 1994). These effects of E_2 require gene transcription and protein synthesis in target HYP-POA neurons that control reproductive function (Pfaff et al., 1994). We have shown that E_2 increases α_{1B}-adrenoceptor mRNA levels and binding density in the HYP-POA (Petitti et al., 1992; Karkanias et al., 1996). We now demonstrate that chronic blockade of brain IGF-IRs with the competitive antagonist JB-1 prevents the E_2-induced increase in the density of α_{1B}-adrenoceptor binding sites in the HYP and POA. JB-1 treatment does not have nonspecific effects on brain NE receptors, because neither basal α_{1B}-adrenoceptor nor α_{1A}-adrenoceptor densities are affected by the drug. It is also unlikely that the actions of JB-1 are attributable to interference with other growth factor signaling pathways (e.g., epidermal growth factor, platelet-derived growth factor), because this peptide analog of IGF-1 does not block the actions of other growth factors (Pietrzkowski et al., 1992).

The mechanism or mechanisms by which IGF-IR and E_2 interact to regulate α_{1B}-adrenoceptor expression in the HYP-POA

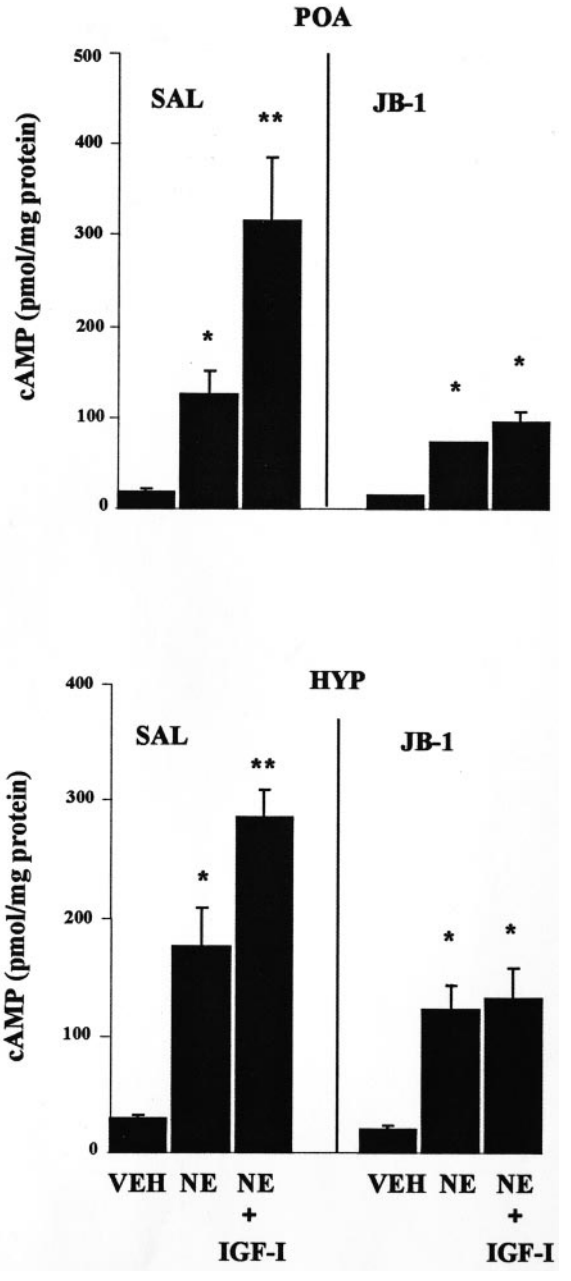

Figure 4. Effect of JB-1 infused into the third ventricle on E_2-dependent, IGF-I enhancement of NE-stimulated cAMP. POA and HYP slices from E_2-treated female rats were prepared from animals infused chronically with JB-1 or saline (*SAL*) as described in Materials and Methods. Slices were incubated for 15 min with 10 nM IGF-I followed by a 20 min incubation with 0.01 N HCl vehicle (*VEH*) or 100 μM NE. The PDE inhibitor 1 mM IBMX was included. The data presented are the means ± SEM from four independent replications. *VEH; **all other groups ($p < 0.05$).

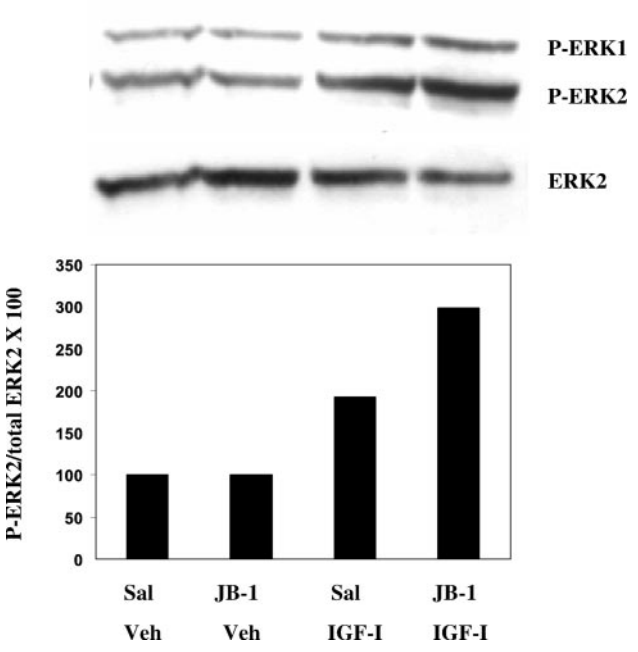

Figure 5. Effect of JB-1 infused into the third ventricle on acute IGF-I activation of extracellular receptor activated kinase 2 (*ERK2*). HYP slices from E_2-treated female rats were prepared from animals infused chronically with JB-1 or saline (*Sal*) as described in Materials and Methods and incubated for 15 min with 10 nM IGF-I or vehicle (*Veh*). Immunoblots were done using a monoclonal antibody for phosphorylated ERK1/2 (P-ERK1/2) and for total ERK2. Phosphotyrosine immunoblots were quantitatively analyzed by taking the ratio of the OD of the P-ERK2 band to the OD of the total ERK2 band. The immunoblot shown is representative of two independent experiments.

remain unknown, although there are several possibilities. Downstream mitogenic signals activated by IGF-IR may increase the expression or transcription activating function of E_2 receptors (Aronica and Katzenellenbogen, 1993; Stoica et al., 2000). Therefore, blockade of IGF-IRs could result in downregulation of either the expression and/or transcription activation function of E_2 receptors. This possibility is consistent with the observation that IGF-IRs are colocalized with both E_2 receptor-α and -β in neurons and glia in various brain regions, including HYP and POA (Cardona-Gomez et al., 2000). Alternatively, IGF-I may promote secretion of other trophic factors or signaling molecules from HYP-POA glial cells in an E_2-dependent manner, resulting in increased expression of α_{1B}-adrenoceptor in the HYP-POA.

The increased expression of α_{1B}-adrenoceptor in the HYP-POA, observed in response to E_2 treatment, is believed to mediate the facilitatory component of NE action on sexual receptivity and LH secretion (Kow et al., 1992; Hosny and Jennes, 1998). Both α_{1B}-adrenoceptor mRNA and protein are expressed in the POA and HYP (Blendy et al., 1990; Pieribone et al., 1994; Acosta-Martinez et al., 1999). In the POA, GnRH cell bodies show the highest density of α_{1B}-adrenoceptor-immunostaining, whereas GnRH nerve terminals in the median eminence show moderate immunostaining (Hosny and Jennes, 1998). Many cells and fibers in the arcuate nucleus–median eminence, the site of GnRH release, demonstrate robust α_{1B}-adrenoceptor immunostaining (Acosta-Martinez et al., 1999). This is also the primary neural site at which IGF-I and E_2 interact to regulate synaptic plasticity (Fernandez-Galaz et al., 1999). The ventromedial HYP, the major site of E_2 facilitation of sexual receptivity, also contains both mRNA and protein for α_{1B}-adrenoceptor (Pieribone et al., 1994; Acosta-Martinez et al., 1999). Thus, E_2 regulation of the hypothalamic-pituitary-gonadal axis and sexual behavior may involve IGF-IR-dependent increases in the expression of α_{1B}-adrenoceptors in target regions of the HYP-POA.

E_2 and IGF-I regulation of LH release

The ability of chronic JB-1 treatment to reduce E_2 and progesterone-dependent increases in plasma LH suggests that IGF-IR blockade suppresses estrogen positive feedback regulation of LH release. JB-1 treatment did not interfere with LH synthesis or release, because JB-1 infusion into OVX rats given no hormone replacement does not reduce the elevated plasma LH levels observed in OVX rats. If JB-1 acts on pituitary gonadotropes to block LH synthesis or secretion, LH levels in the OVX control rats infused with JB-1 should have been reduced. This was clearly not the case. Nonetheless, it is possible that treatment with the IGF-IR antagonist influenced pituitary sensitivity to GnRH. Future experiments could evaluate this issue by measuring the LH response to exogenously administered GnRH in JB-1-infused animals.

Although we cannot rule out the possibility that the IGF-IR antagonist interferes with progesterone action as well, it is more likely that JB-1 acted primarily to interfere with estrogen-positive feedback. First, progesterone facilitation of LH release requires E_2 priming; in the absence of previous exposure to estrogen, progesterone inhibits LH secretion (Freeman, 1994). Second, at the time blood samples were taken from E_2-treated rats infused with saline, plasma LH levels were not significantly lower than in OVX control animals (Fig. 2). Blood was collected 48 hr after the first estrogen injection, ~9 hr into the dark phase of the reverse light/dark cycle. Thus, it is likely that the LH values observed in E_2-treated, saline-infused rats reflect positive feedback caused by estrogen alone. Plasma LH levels in E_2-treated rats were significantly reduced by JB-1, suggesting that antagonism of IGF-IR activity influences estrogen-positive feedback.

E_2-induced synaptic remodeling in the arcuate nucleus, which is also dependent on IGF-IR activity (Fernandez-Galaz et al., 1999), is believed to be critical for the preovulatory release of GnRH (Perez et al., 1993; Garcia-Segura et al., 1994). Thus, JB-1 treatment in our experimental paradigm may also inhibit E_2-induced synaptic remodeling in the arcuate nucleus, resulting in inhibition of E_2-induced positive feedback on GnRH release, and ultimately inhibition of LH release. Our results add to an increasing body of evidence suggesting that IGF-I and E_2 work together to modulate LH secretion. Furthermore, they implicate the α_{1B}-adrenoceptor as a mediator of E_2/IGF-I enhancement of gonadotropin release.

Interaction between IGF-I and E_2 may also be involved in changes in estrogen-negative feedback believed to be essential for the initiation of female puberty. Peripheral administration of IGF-I to OVX, adolescent female monkeys attenuates E_2 negative feedback on LH release (Wilson, 1995). Likewise, intracerebroventricular administration of IGF-I enhances LH release in both juvenile and peripubertal female rats, accelerating the initiation of puberty (Hiney et al., 1996).

E_2 and IGF-I regulation of reproductive behavior

IGF-IR blockade during E_2 priming partially attenuates E_2-dependent sexual behavior. The effects of JB-1 on lordosis are caused by blockade of IGF-IRs during E_2 priming rather than by residual JB-1 remaining in the brain after multiple infusions. This

conclusion is supported by the observation that acute intracerebroventricular infusion of JB-1 between 4 and 12 hr before behavior testing has no effect on lordosis. We also showed that infusion of 8-bromo-cGMP, a cell-permeable analog of cGMP, reverses the partial inhibition of sexual behavior induced by multiple intracerebroventricular infusions of JB-1. This observation is in accordance with previous results from our laboratory demonstrating that inhibition of sexual behavior by systemic administration of an α_1-adrenoceptor antagonist is reversed by intracerebroventricular administration of 8-bromo-cGMP (Chu and Etgen, 1999). Moreover, administration of progesterone to E_2-primed rat switches α_1-adrenoceptor signaling from phospholipase C activation and potentiation of adenylyl cyclase activity to stimulation of nitric oxide-dependent activation of cGMP synthesis (Chu and Etgen, 1999; Chu et al., 1999). Hence, the ability of 8-bromo-cGMP to rescue the partial inhibition of E_2-dependent sexual behavior by JB-1 indirectly suggests that interference with α_1-adrenoceptor signaling pathways may be responsible for the observed lordosis inhibition.

There are several reasons why JB-1 may produce only partial attenuation of sexual behavior. First, It is possible that multiple intracerebroventricular infusions of JB-1 did not completely block brain IGF-IRs. Therefore, future experiments using higher doses or constant intracerebroventricular delivery of JB-1 might produce a more complete inhibition of lordosis. Second, JB-1 treatment blocks the E_2-dependent increase of α_{1B}-adrenoceptor binding without affecting basal α_{1B}-adrenoceptor in the HYP-POA. Hence, the remaining α_{1B}-adrenoceptor present might be sufficient to support partial expression of sexual behavior. Third, E_2 is thought to produce maximal sexual receptivity in part by induction of progesterone receptor expression in the HYP (MacLusky and McEwen, 1978; Blaustein, 1982). Thus, JB-1 administration may have incompletely blocked E_2 induction of progesterone receptors in the HYP. Fourth, because the survival of species is dependent on successful reproduction, there are likely to be redundant neural elements (neurotransmitters, neurohormones and neuropeptides) on which E_2 and progesterone act to coordinate reproductive physiology. Therefore, IGF-IRs may mediate the actions of E_2 on only a subset of these neural targets of hormone action.

E_2 and IGF-I modulation of α_1-adrenoceptor signaling

Previously, we demonstrated that acute application of IGF-I onto HYP and POA slices *in vitro* enhances NE-stimulated cAMP accumulation, via α_1-adrenoceptor potentiation of adenylyl cyclase activation, only in E_2-primed female rats (Quesada and Etgen, 2001). We now show that blockade of IGF-IR activation during E_2 priming prevents both the increased expression of α_{1B}-adrenoceptors and IGF-I enhancement of NE-stimulated cAMP accumulation in the HYP and POA. Thus, the E_2-dependent effect of acute application of IGF-I on α_1-adrenoceptor signaling, like E_2 induction of α_{1B}-adrenoceptors and LH release, relies on brain IGF-IR activity during E_2 priming. The E_2 dependence of IGF-I potentiation of NE-stimulated cAMP synthesis might be attributable to the induction of α_{1B}-adrenoceptor expression in HYP and POA cells that also express IGF-IRs. Because *in vitro* application of IGF-I onto HYP slices from JB-1-infused rats induces ERK1/2 phosphorylation, the effects of JB-1 on NE signaling must be caused by blockade of IGF-IR during E_2 priming rather than residual JB-1 remaining in the brain after the multiple intracerebroventricular infusions. In addition, E_2 can increase ^{125}I-IGF-I binding density (Quesada and Etgen, 2001) and IGF-IR content in the HYP (Michels et al., 1993; Pons and Torres-Aleman, 1993; Wimalasena et al., 1993). Therefore, E_2 dependence of the interaction between IGF-I and NE may involve upregulation of IGF-IR, the α_{1B}-adrenoceptor or both.

In summary, these data indicate that brain IGF-IR activity is necessary for long-term effects of E_2 on α_{1B}-adrenoceptor expression and function in the HYP and POA as well as for hormone-dependent sexual receptivity and positive feedback regulation of LH release. These results demonstrate a novel mechanism by which changes in noradrenergic signal transduction resulting from E_2 and IGF-I action in the brain control GnRH release and the expression of reproductive behavior.

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