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Thyroid hormone and estrogen interact to regulate behavior

(reproduction/hypothalamus/steroid receptors)

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ABSTRACT **Environmental perturbations that increase plasma thyroid hormone (T_3) concentrations also profoundly affect female reproductive behavior and physiology. We explored whether these effects were mediated by interactions between T_3 receptor (TR) and estrogen receptor (ER). This hypothesis was of interest because the half-site of a consensus T_3 response element DNA sequence is identical to an ER response element (ERE), and TRs bind to a consensus ERE. Molecular data presented in the accompanying paper [Zhu, Y.-S., Yen, P. M., Chin, W. W. & Pfaff, D. W. (1996) *Proc. Natl. Acad. Sci. USA* 93, 12587–12592] demonstrate that TRs and ERs are both present in rat hypothalamic nuclear extracts and that both can bind to the promoter the hypothalamic gene preproenkephalin and that interations between liganded TRs and ERs affect preproenkephalin transcription. In this paper, we show that molecular interactions between TRs and ERs are sufficient to mediate environmental effects on estrogen-controlled reproductive behavior. Ovariectomized (OVX) rats treated with high doses of T_3 showed significantly lower levels of lordosis behavior in response to estradiol benzoate (EB) compared with OVX females treated with EB alone. Conversely, thyroidectomized/OVX females treated with EB showed significantly greater levels of lordosis behavior compared with OVX females treated with EB, showing the effect of endogenous T_3. Thyroid hormone interference with EB-induced behavior could not be explained by a reduction in plasma E_2 concentrations or by a general reduction in responsiveness of EB-sensitive tissues. Moreover, numbers of hypothalamic ER-immunoreactive cells increased dramatically following T_3 treatment. These data suggest that T_3 may reduce EB-dependent sexual behavior through interactions between TR and ER in the nuclei of behaviorally relevant hypothalamic neurons, envisioning for the first time a functional consequence of interactions between two nuclear hormone receptors in brain. These results also open up the possibility of molecular interactions on DNA encoding environmental signals, a new field for the study of neuronal integration.**

The half-site of a consensus sequence of nucleotide bases constituting a thyroid hormone response element is identical to an estrogen receptor (ER) response element (ERE) half-site, and thyroid hormone receptors (TR) can bind to a consensus ERE (1). Manipulations of thyroid hormones and estrogen *in vitro* potentiate or mutually inhibit effects on gene expression (2–4). Our data presented in the accompanying paper (5) demonstrates that both TRs and ERs are present in rat hypothalamic nuclear extracts and both can bind to a preproenkephalin (PPE) promoter ERE. Using slot-blot hybridization, we also found that ovariectomized (OVX) rats treated with high doses of triiodothyronine (T_3) in combination with estradiol benzoate (EB) had significantly less PPE mRNA in the hypothalamus compared with females treated with EB alone. Taken together with data from other systems (1–4), our molecular results suggest that there may be competition between TR and ER for hormone response element binding of hypothalamic genes, such as PPE.

Such interactions between nuclear receptors could subserve a new level of neuronal integrations. For example, one consequence of adverse environmental conditions, such as cold temperature, is altered plasma concentrations of thyroid hormones, and such changes in climate also profoundly affect female mammalian reproductive physiology and behavior (6–9). Decreased ambient temperature can delay puberty in juveniles, and inhibit ovulation, decrease sexual behavior, alter gonadotropin release, and decrease litter size in adults (10–12). Thus, changes in serum thyroid hormone concentrations might be important for signaling environmental conditions to neuroendocrine mechanisms, conceivably by interactions of TRs with ERs on hormone response elements of relevant genes (13). The neural circuitry involved in lordosis, a female rat sexual behavior, is relatively well-understood and is known to be mediated by estrogen acting via hypothalamic ERs (14). However, interactions between thyroid hormones and estrogen on reproductive behavior have not been studied.

MATERIALS AND METHODS

Animals and Experimental Design. In the first experiment, we asked whether altered plasma thyroid hormone levels would affect EB-induced lordosis behavior. We used both OVX and thyroidectomized/ovariectomized (TX/OVX) Sprague Dawley rats for this study. We gave daily low doses of EB (2 μg, s.c.) to slowly increased plasma estradiol (E_2) concentrations in OVX rats, thereby gradually increasing sexual receptivity. T_3 was given in high doses (500 μg per kg body weight, i.p.) to OVX females to induce hyperthyroid plasma concentrations. In contrast, lower doses of T_3 (20 μg per kg body weight, i.p) were given to TX/OVX females to increase plasma concentrations within the physiological range.

Two weeks after surgery, OVX rats (175–225 g) were treated daily for 10 days with vehicle (n = 13), EB (2 μg, s.c.; n = 13), high doses of T_3 (500 μg per kg body weight, i.p.; n = 13), or EB + high doses of T_3 (n = 15). TX/OVX females were treated daily for 10 days with vehicle (n = 14), EB (2 μg; n = 16), low doses of T_3 (20 μg per kg body weight i.p.; n = 13), or low doses of T_3 + EB (n = 15). T_3 was administered every 12 hr at 9:00 a.m. and 9:00 p.m., and EB was injected once a day at 9:00 a.m. In both experiments, animal surgeries were performed by the supplier (Charles River Breeding Laboratories) and animals were maintained on a 12-hr light/12-hr dark cycle (lights on at 7:00 p.m.). Food and water were

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Abbreviations: ER, estrogen receptor; ERE, ER response element; TR, thyroid hormone receptor; PPE, preproenkephalin; OVX, ovariectomized; T_3, triiodothyronine; EB, estradiol benzoate; TX, thyroidectomized; LQ, lordosis quotient; E_2, estradiol; IR, immunoreactive; VMH, ventromedial hypothalamus.

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provided ad lib, and TX/OVX females received 1% calcium chloride in their drinking water.

Females were tested for sexual receptivity as described below every other day beginning on day 2 for a total of five tests. All females were anesthetized and killed either by decapitation or by perfusion on the afternoon of the final behavior test (day 10). Blood samples were collected from either trunk blood (decapitated animals) or cardiac puncture (perfused animals). Samples were centrifuged, plasma frozen, and stored at −20°C until assayed for T_3 and/or E_2 concentrations. The uterus was removed and immediately weighed when the rat was killed. One uterine horn was also placed in Bouins' fixative and processed for paraffin histology as described below.

In the second experiment, we asked whether pretreatment with T_3 would affect female rat sexual behavior in response to acute EB treatment. Two weeks after surgery, OVX females were treated daily for 7 days with high doses (500 μg per kg body weight, i.p.) of either T_3 ($n = 21$) or vehicle ($n = 15$). T_3 was administered every 12 hr at 9:00 a.m. and 9:00 p.m. On days 5 and 6 (between 9:00 and 10:00 a.m.), 10 μg of EB was administered s.c. to seven vehicle-treated (OVX-EB group) and nine T_3-treated (OVX-EB + T_3) females. Twenty-four hours after the second EB injection, females were tested for sexual receptivity with a sexually experienced male as described below. On day 7 after the behavior test, females were anesthetized and killed either by decapitation or by perfusion. The uterus was removed and immediately weighed when the rat was killed.

Sexual Behavior. Females were tested for sexual behavior with sexually experienced males between 12:00 and 3:00 p.m. under red lights. Each test lasted for either 10 min or until the male mounted 10 times. Lordotic responses of the female to male mounting, intromitting and ejaculation were recorded by an observer blind to the experimental treatment of the female. Lordosis quotient (LQ) equals the total number of lordosis responses/total number of mounts multiplied by 100 (L/M × 100).

Radioimmunoassay. Plasma aliquots (100 μl) were assayed for total T_3 using a highly sensitive commercially available kit [DPC (Los Angeles) Total T_3 Coat-a-Count] that was adapted for use with rat serum according to the companies instructions. For E_2 assay, duplicate aliquots (500 μl) were extracted with diethyl ether. The extracts were dried and reconstituted, and 17 β-E_2 was measured using the Collins TGK anti-estradiol-6-BSA antibody as previously described (15).

Uterine Tissues. In the first study, one uterine horn was placed in Bouins' fixative for 24 hr at 4°C, dehydrated in a series of alcohols, cleared in butanol, and embedded in paraffin. Ten-micrometer coronal and sagittal sections were stained with hematoxylin and eosin. Coronal sections were projected (with a Bausch & Lomb microprojector) onto tracing paper, and endometrial wall thickness was measured at three different regions of each uterus to obtain the average endometrial thickness for each female.

Immunocytochemistry. In the first study, three females per group ($n = 24$) were perfused through the aorta with saline followed by 4% paraformaldehyde with 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Every sixth 30-μm frozen section was collected into cold 0.05 M TBS, pH 7.2, and processed for ER-immunoreactive (IR) using H222 (generously provided by Dr. G. Greene, University of Chicago). Sections were sequentially incubated in 1% borohydride, 0.1% hydrogen peroxide, and 0.1% gelatin containing 10% normal goat serum and 1% Triton X-100. Sections were then incubated for 36–48 hr at 4°C in primary antibody (H222 = 3 μg/ml). Sections were processed with a rat IgG ABC Elite kit (Vector Laboratories) and immunoreactivity was visualized with diaminobenzidine. Each immunocytochemical run included at least one brain from each treatment group. ER IR cells were counted in every sixth section (right and left side) throughout the entire rostral-caudal extent of the VMH.

Statistical Analysis. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls comparison of groups; $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

In the first experiment, we examined the effects of altered plasma T_3 levels on lordosis behavior in both OVX and TX/OVX rats (Fig. 1). In OVX females, daily treatment with high doses of T_3 (500 μg per kg body weight per day) for 10 days induced hyperthyroid plasma concentrations (7). Low doses of EB (2 μg per day) were given to slowly increase circulating E_2 concentrations during the 10 days (Table 1), which resulted in a gradual rise in lordosis behavior in OVX females (Fig. 1*A*). During the initial three behavior tests, mean LQ scores were equivalent in EB-treated females regardless of T_3 treatment (Fig. 1*A*). However, during tests four and five, when mean LQ scores were above 50, hyperthyroid females had significantly lower lordotic responses (Fig. 1*A*). Hyperthyroid females were physically healthy and interacted continuously with males during all five behavior tests. In fact, the decrease in LQ during the fourth and fifth tests was due to increased rejection behaviors rather than a decrease in male mount attempts. Males attempted to mount hyperthyroid EB-treated females an average of 17 times per 10 minute test (tests four and five combined), but females displayed rejection behaviors (rolling on back, fighting, or kicking) during 38% of these mount attempts. In contrast, OVX females treated with EB alone only displayed rejection behaviors in response to 0.2% of mount attempts during tests four and five. OVX females treated with either vehicle or T_3 alone did not demonstrate any lordosis behavior.

In contrast, thyroidectomy dramatically elevated LQ scores in OVX females treated with EB by the second behavior test (Fig. 1*B*). Interestingly, TX/OVX females treated with low doses of T_3 (20 μg per kg body weight per day) in combination with EB had mean LQ scores equivalent to females treated with EB alone on tests two and three. Therefore, only on test four was there a significant difference between these groups (Fig. 1*B*). At the time of sacrifice, females treated with low T_3 had plasma concentrations within the physiological range (Table 1; refs. 16 and 17). Other studies have indicated that several days of T_3 treatment are required to restore serum concentrations after thyroid removal, as well as to induce hyperthyroidism in thyroid-intact rats (18–20). This may explain why our behavioral effects were not evident until the final two tests in both OVX and TX/OVX females (Fig. 1 *A* and *B*).

A direct comparison of the lordosis behavior results (same data presented in Fig. 1 *A* and *B*) between OVX and TX/OVX females in response to EB treatment clearly demonstrates the role of endogenous T_3. In the absence of the thyroid, OVX females became sexually receptive significantly sooner (Fig. 1*C*). During the second test, all TX/OVX animals treated with EB had an LQ of 70% or greater. By the third test (day 6), 13 of 16 of these females had an LQ of 100%, as compared with only 3 of 13 OVX + EB females. During the final behavior test, EB-treated OVX and TX/OVX females had equivalent mean LQ scores, suggesting that continued EB treatment eventually overcomes the effect of endogenous T_3 (Fig. 1*C*). These data suggest that once plasma estrogen levels are elevated above some threshold level, endogenous T_3 can no longer inhibit the behavioral effects. Finally, Fig. 1*D* (data from Fig. 1 *A* and *B*) shows that EB treatment induced higher levels of lordosis in TX/OVX females given low doses of T_3 than in hyperthyroid OVX females.

A second behavioral experiment was designed to ask whether pretreatment with high doses of T_3 (500 μg T_3 per kg

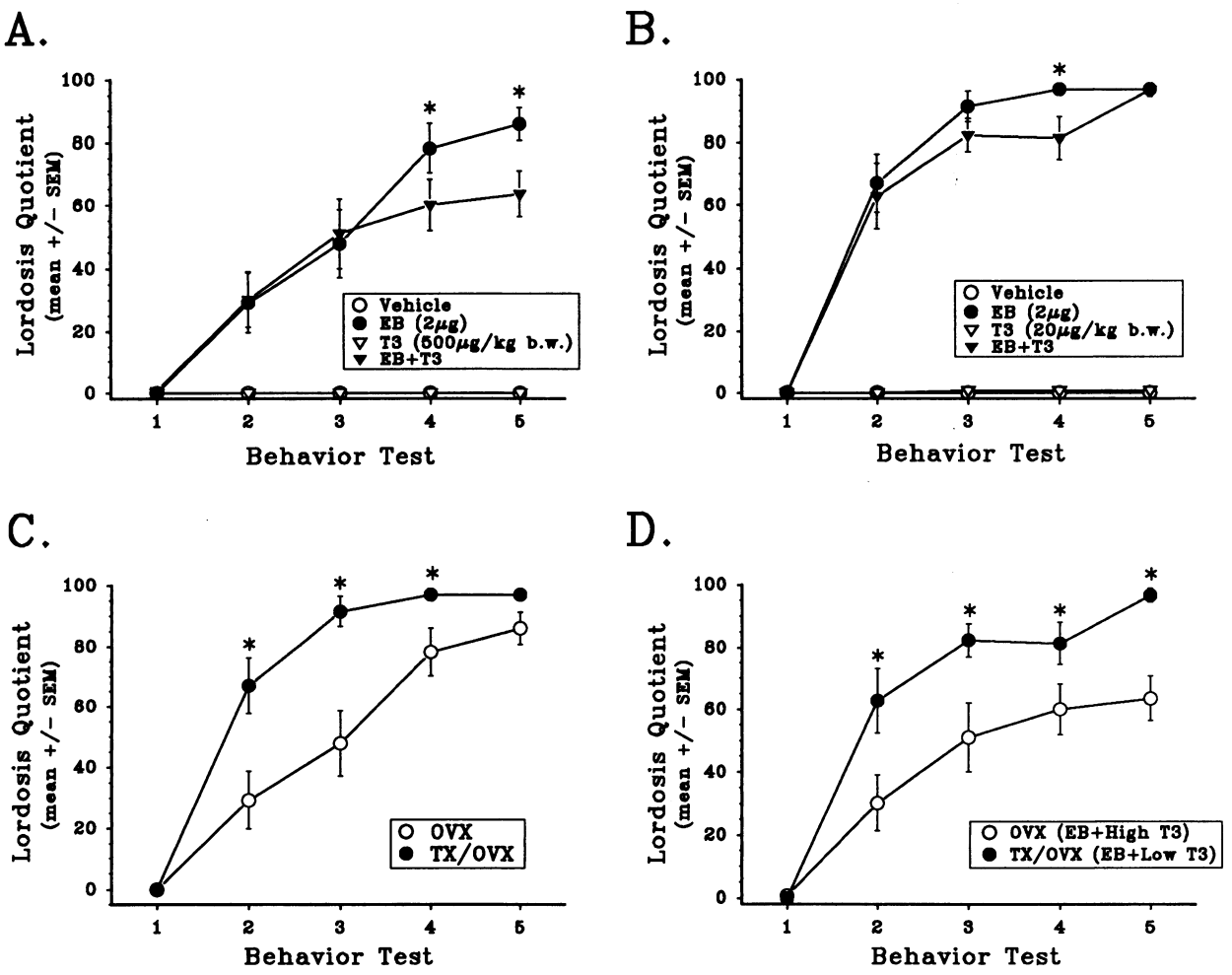

FIG. 1. Effect of altered circulating levels of T_3 on EB-induced sexual behavior in OVX and TX/OVX females. (*A*) Mean LQ of OVX rats treated daily for 10 days with vehicle ($n = 13$), EB ($n = 13$), high doses of T_3 ($n = 13$), or EB + T_3 ($n = 15$). Sexual behavior tests were conducted and scored as described. *, Significantly different from OVX females treated with EB + T_3, $P < 0.05$. (*B*) Lordosis behavior demonstrated by OVX/TX females treated with vehicle ($n = 14$), EB ($n = 16$), low doses of T_3 ($n = 13$), or EB + T_3 ($n = 15$). *, Significantly different from OVX/TX females treated with EB + T_3, $P < 0.05$. (*C*) Direct comparison of mean LQ scores (data from *A* and *B*) between OVX and OVX/TX rats to reveal the effect of endogenous T_3. *, Significantly different, $P < 0.05$. (*D*) Direct comparison of mean LQs (data from *A* and *B*) of rats treated with EB and either with high doses of T_3 (OVX) or with low (physiological) doses of T_3 (OVX/TX). *, Significant difference, $P < 0.05$.

body weight per day) would alter lordosis behavior in response to acute EB treatment in OVX rats. Ovariectomized rats were treated for 4 days with T_3 alone before administration of EB (10 μg per rat). EB injections were given 24 and 48 hr before a single behavior test. As in the first study, hyperthyroid females treated with EB had significantly lower levels of lordosis than OVX females treated with EB alone (Fig. 2). Taken together, these behavioral data show that high circulating concentrations of T_3 interfere with EB-induced lordosis in OVX rats. Moreover, in the complete absence of peripheral T_3, OVX females are more sensitive to EB treatment.

Table 1. Plasma hormone concentrations

Condition	Total plasma T3, ng/dl	Plasma E_2, pg/ml
OVX-Veh	35.4 ± 2.6 (8)	11.6 ± 1.9 (8)
OVX-EB	50.6 ± 5.0 (6)	23.9 ± 2.6 (8)
OVX-T3	2074 ± 437.4 (9)	11.9 ± 2.8 (6)
OVX-EB+T3	1631.6 ± 418 (9)	29.2 ± 2.3 (14)
TX/OVX-Veh	<25 (9)	12.1 ± 2.1 (10)
TX/OVX-EB	<25 (9)	32.5 ± 2.1 (11)
TX/OVX-T3	135 ± 16.6 (13)	9.5 ± 1.8 (10)
TX/OVX-EB+T3	170.9 ± 28.4 (12)	32.4 ± 3.1 (7)

Data are expressed as mean ± SEM. Numbers in parentheses indicate number of animals per group.

Although these behavioral effects may be attributed to molecular mechanisms involving protein–DNA interactions or protein–protein interactions between ER and TR, other explanations were possible. Therefore, additional experiments were performed to examine other potential mechanisms. For example, T_3-induced changes in absorption, distribution, and/or clearance of the injected EB could have altered the behavioral responses. However, when plasma E_2 radioimmunoassay levels were measured after 10 days of EB treatment, E_2 levels were equivalent in hypo- and hyperthyroid females (Table 1). Moreover, these concentrations were within the physiological range for early proestrus female rats (21). In addition, the uterus, an estrogen-sensitive tissue, increased in both wet weight and endometrial thickness in all EB-treated females, regardless of circulating concentrations of T_3 (Fig. 3). Therefore, pharmacokinetics alone cannot account for the behavioral responses of these females.

It was also possible that elevated serum T_3 levels could reduce neural ER concentration and thereby alter EB-induced sexual behavior. Since estrogen promotes lordosis by acting primarily on ER-containing neurons within the ventromedial hypothalamus (VMH; refs. 14, 22, and 23), we examined ER-IR cell number within this nucleus in three animals per group from the first behavioral experiment (Fig. 4). OVX and TX/OVX females had equivalent numbers of ER-IR neurons

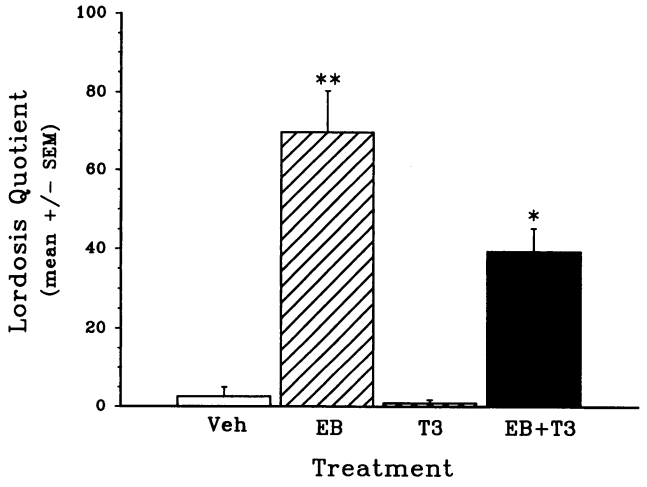

FIG. 2. Effect of pretreatment with T_3 on EB-induced sexual behavior. (*A*) Mean LQ scores of females treated with vehicle (n = 8), EB (10 μg s.c. 24 and 48 hr before behavior test; n = 7), T_3 (500 μg per kg body weight i.p. for 7 days; n = 12), or EB + T_3 (n = 9). Sexual behavior tests were conducted and scored as described. *, Significantly different from vehicle- and T_3-treated females; **, significantly different from all other groups ($P < 0.05$).

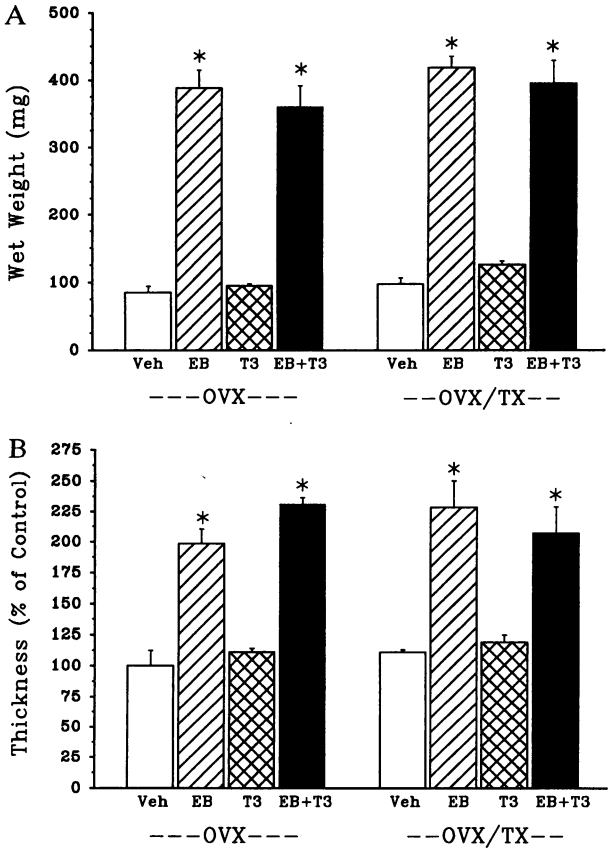

FIG. 3. Changes in uterine wet weight and endometrial thickness after 10 days of hormone treatment. (*A*) Uterine wet weight in OVX and TX/OVX females treated for 10 days with vehicle (n = 4 and 5), EB (n = 5 and 6), T_3 (n = 7 and 5), or EB + T_3 (n = 7 and 6). *, Significantly different as compared with vehicle- and T_3-treated females. (*B*) Percent change in endometrial wall thickness (compared with vehicle-treated OVX females) after 10 days of treatment with vehicle (n = 4 and 3), EB (n = 3 and 4), T_3 (n = 3 and 3), or EB + T_3 (n = 4 and 4). *, Significantly different from vehicle- and T_3-treated females.

in the VMH (Fig. 4*A*), indicating that endogenous circulating T_3 was not reducing neural ER-IR. Treatment with EB decreased ER-IR cell number in OVX females, extending earlier mRNA and immunocytochemical findings (24–26). However, this decrease was much larger in TX/OVX females, where few ER-IR cells were seen in the VMH following EB treatment (Fig. 4 *A*, *D*, and *E*). Surprisingly, 10 days of treatment with either high doses of T_3 in thyroid-intact or low doses of T_3 in TX/OVX females resulted in dramatic increases in ER-IR cell number in the VMH (Fig. 4 *A*, *F*, and *G*). This increase occurred with or without EB treatment (Fig. 4 *H* and *I*). Similar unexpected changes in ER-IR concentration were observed throughout the brains of these females (see Fig. 4 *B–I*). A change in immunoreactive ER distribution was also noted in the VMH of T_3 treated animals (Fig. 4 *F–I*). In rats, ER-containing neurons are predominantly in the ventrolateral region of the VMH (22–26). In our study, immunoreactive neurons were always most dense in this region, but ER-IR cells extended more dorsally in T_3 treated than in vehicle- or EB-treated rats (Fig. 4 *B–I*). Similar increases in ER-IR were noted in the VMH of T_3 treated females in the second behavioral experiment (unpublished data).

The mechanism(s) responsible for the dramatic changes in ER-IR in the present study are still unknown and are currently under investigation. One possibility is that the ER antibodies used in this study preferentially bind to unliganded ERs, as suggested by other investigators (24). In this case, our data might suggest that exogenous T_3 treatment not only up-regulated ER-IR cells, but also interfered with estrogen-ER binding. For example, animals treated with either T_3 alone or in combination with EB had large numbers of ERs throughout the brain. However, there was no correlation between numbers of ER-IR cells and reproductive behavior. Lordosis scores on the final test were significantly different between OVX and TX/OVX animals treated with EB + T_3 (Fig. 1*A*), but ER-IR cell numbers were dramatically elevated in both groups. The presence or absence of a thyroid did not alter ER-IR cell numbers (OVX and OVX/TX vehicle-treated), suggesting that basal levels of ER are maintained after thyroidectomy. The fact that very few ER-IR neurons were present following EB treatment in TX/OVX animals again suggests that in the absence of T_3 more ER binding occurred. Regardless of the mechanism(s) involved, these data indicate that T_3 effects on hypothalamic ER-IR concentration were in the wrong direction to explain our behavioral data.

Since these physiological mechanisms could not account for our behavioral results, we retain the hypothesis that TR/ER interactions in brain might be operative. The rat medial hypothalamus has both ER- and TR-containing neurons in overlapping regions, including the VMH (22, 23, 27–29). Therefore, we are presently examining changes in EB-induced hypothalamic gene expression due to altered plasma T_3 concentrations (30–32). PPE and progesterone receptors have been demonstrated to play a role in the regulation of female rat sexual behaviors (33–35), and mRNA of both genes increase in the VMH after EB treatment in OVX rats (36–38). As shown in the accompanying paper (5), both ER and TR can bind to the PPE promoter (5) and Scott *et al.* (32), in our lab, found that TR can also bind to the progesterone receptor promoter. In addition, liganded TRs interfere with estrogen-ER induction of transcription through the PPE promoter in *in vitro* transfection assays. Using *in situ* (30) and slot-blot hybridizations (5), we also found that T_3 attenuated the EB-induced increase in PPE mRNA in the hypothalamus of OVX rats. These results suggest that TRs have the ability to compete with ER on estrogen-responsive genes in the rat hypothalamus, as previously reported in *in vitro* systems (2–4).

While it is clear that competition for DNA binding sites or other molecular interactions between ER and TR within hypothalamic cell nuclei would be sufficient to explain our

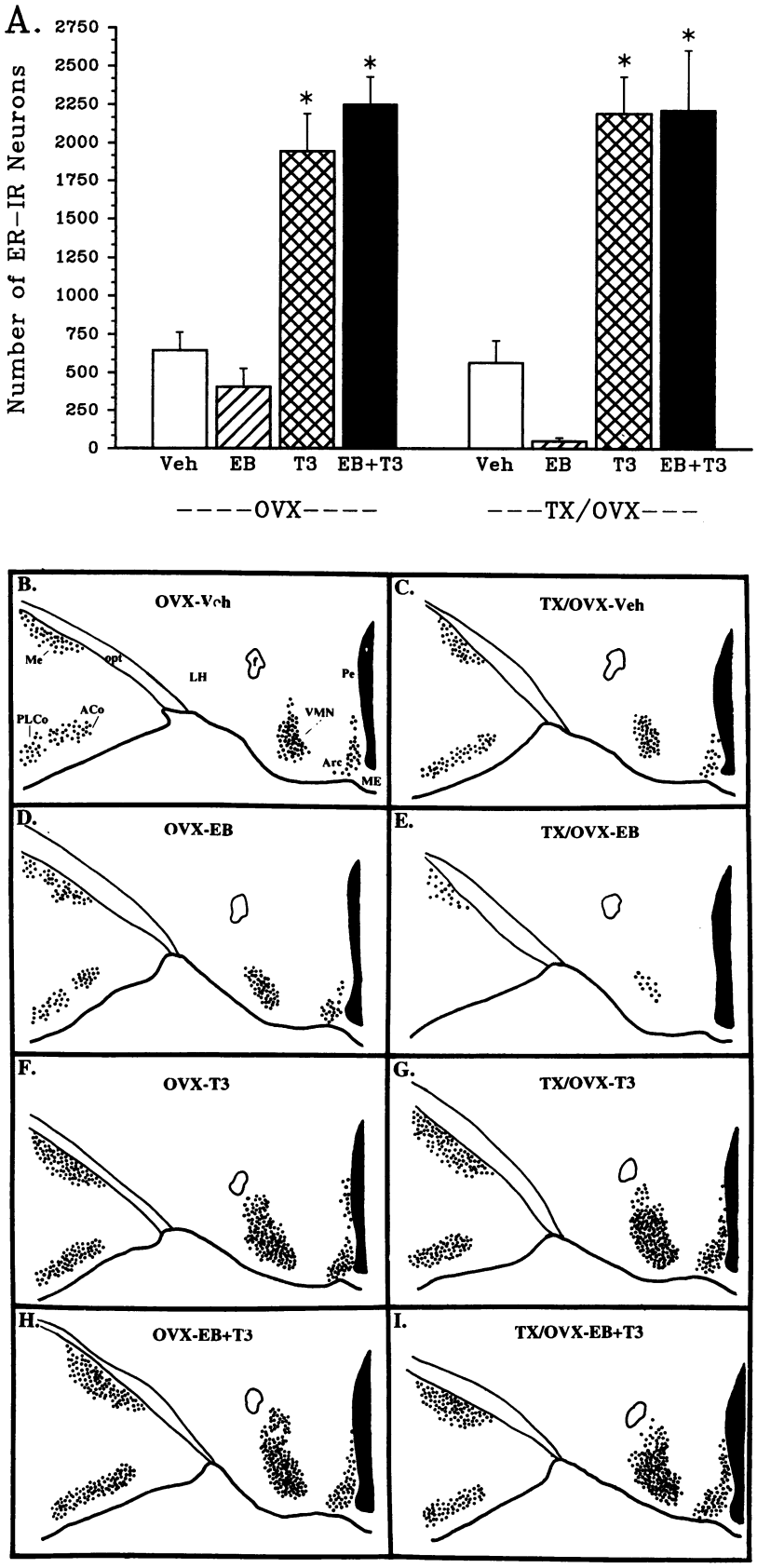

FIG. 4. Effect of T_3 and EB on ER-IR cell number and distribution in the VMH. (*A*) Mean number of ER-IR neurons in the VMH after 10 days of hormone treatment in OVX and OVX/TX rats (n = 3 per group). *, Significantly different from vehicle and EB treated females. (*B–I*) Camera lucida drawings of representative 30 μm sections of OVX and TX/OVX females (hormone treatment indicated on each drawing) showing ER-IR neurons in the VMH, arcuate nucleus (ARC), and amygdala. Each circle represents a ER-IR neuron. ACo, anterior cortical amygdaloid nucleus; f, fornix; LH, lateral hypothalamus; Me, medial amygdala; ME, median eminence; opt, optic tract; PLCo, posterolateral cortical amygdaloid nucleus; Pe, periventricular nucleus; V, third ventricle.

behavioral data, other mechanisms including effects on other neurons along the lordosis circuit are also possible. In any case, such interactions among hormone systems are clearly biologically important. Clinically, hyperthyroidism causes physiological and behavioral changes in women. Patients experience decreased libido, increased nervousness and irritability, and altered menstrual cycles and decreased fertility (39). In more fundamental terms, integrating molecular knowledge with environmental thought and neurobiological technique has likely revealed for the first time, to our knowledge, a behavioral consequence of interactions between nuclear hormone receptors in brain.

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