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The Distribution of Estrogen Receptor-β mRNA in Forebrain Regions of the Estrogen Receptor-α Knockout Mouse

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Abstract Neurons in the hypothalamus of estrogen receptor α-knockout (ERαKO) mice have been shown to concentrate radiolabeled estrogen and estrogen treatment regulates the expression of progesterone receptor mRNA. The purpose of the present study was to utilize *in situ* hybridization histochemistry to determine the anatomical distribution of ERβ mRNA in ERαKO mouse forebrain. The results of these studies revealed an extensive distribution of ERβ mRNA in the hypothalamic regions including medial preoptic area, suprachiasmatic nucleus, paraventricular nucleus, dorsomedial nucleus, medial tuberal nucleus, and the premammillary nuclei. Additional labeled perikarya were also detected in the glomerular layer of the olfactory bulb; tenia tecta; anterior septum; bed nucleus of the stria terminalis; medial, basolateral and cortical nuclei of the amygdala; cerebral and entorhinal cortex; the septohippocampal nucleus; Ammon's horn of the hippocampus and the dorsal raphe. The results of these *in situ* hybridization histochemical studies have provided novel information about the distribution of ERβ mRNA in the ERαKO mouse forebrain. In addition, these morphological data provides evidence that estrogen may exert its actions in the ERαKO mouse brain via ERβ and thereby maintain organizational and activational effects.

Introduction

The actions of estrogen on the developing and adult brain are thought to be mediated by the classical estrogen receptor (ERα). The development and characterization of an estrogen receptor α-knockout (ERαKO) mouse (1) has shown that ERα is important in rodent procreation, with the ERαKO mice failing to show reproductive behavior (2) and a normal estrous cycle (1). Interestingly, our laboratory has shown that neurons in the ERαKO mouse brain, including cells in the preoptic area of the hypothalamus, have a nuclear uptake and retention of radiolabeled estrogen (3). Moreover, *in situ* hybridization studies have demonstrated that estradiol modulates the expression of progesterone receptor (PR) mRNA in the preoptic area of the ERαKO mouse hypothalamus (3), suggesting that estrogen is still capable of modulating the expression of certain genes in the ERαKO mouse brain.

Recently, Kuiper and colleagues (4) cloned a novel estrogen receptor, designated ER-β. Analysis of the distribution of ER-β mRNA (5,6) and protein (7) in the rat hypothalamus and central nervous system revealed that ER-β mRNA was present in many brain regions including the preoptic, supraoptic and paraventricular nuclei of the hypothalamus; bed nucleus of the stria terminalis; amygdala; cortex; and hippocampus. The localization of ER-β mRNA in the rat preoptic area indicates that estrogen may regulate genes in the ERαKO mouse brain by interacting with ER-β. With the cloning of the mouse ERβ cDNA (8), it is now possible to investigate the localization of ER-β mRNA in the ERαKO mouse brain using *in situ* hybridization.

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Materials and Methods

Animals: C57BL/6J ERαKO mice (1) were genotyped using PCR analysis of tail samples as described (1). 60-day-old ERαKO mice (n=6), housed in the Wyeth-Ayerst animal care facility (AAALAC certified) with a 12-h light/dark photoperiod and free access to tap water and rodent chow, were ovariectomized for 14 days and then exposed to a lethal dose of CO_2. The brains were removed, frozen on dry ice and stored at -80°C until sectioning. Cryostat sections (20μm) were collected on gelatin-coated slides and processed as previously described (9). The studies described in this paper were approved by the Radnor Animal Care and Use Committee at Wyeth-Ayerst Research.

In situ hybridization: A fragment (bases 1484-2083) of the mouse ERβ cDNA (8) 3' untranslated region was amplified from mouse genomic DNA with PCR. The fragment was subcloned into the pGEM-T-Easy vector and verified by sequencing. The resulting plasmid, ERβ-600, was linearized with Nde I (antisense) or Sac II (sense, control) and used to generate ^{35}S-UTP-labeled probes for *in situ* hybridization. Since previous studies (5) revealed that cocktailing two unique riboprobes greatly enhances the hybridization signal, the ERβ-600 probe was evaluated with or without the addition of ERβ-558 (5), a probe complimentary to the 5' region of the rat ERβ cDNA. The results of these studies revealed that both probes had the same distribution in ERαKO mouse tissue and that a ERβ-600/ ERβ-558 cocktail gave the best signal to noise ratio. Therefore, the results described herein used the ERβ-600/ ERβ-558 mixture to evaluate the distribution of ERβ mRNA in the brain. Processed section-mounted slides were hybridized with 200μl of an antisense or sense (control) riboprobe ($8x10^6$ DPM/ probe/ slide) -50% formamide hybridization mix and incubated overnight at 55°C in a chamber humidified with 50% formamide/ 600 mM NaCl. The slides were then rinsed (2xSSC/10 mM Dithiothreitol), treated with RNase A (20μg/ml) and washed at 67°C in 0.1xSSC to remove nonspecific label.

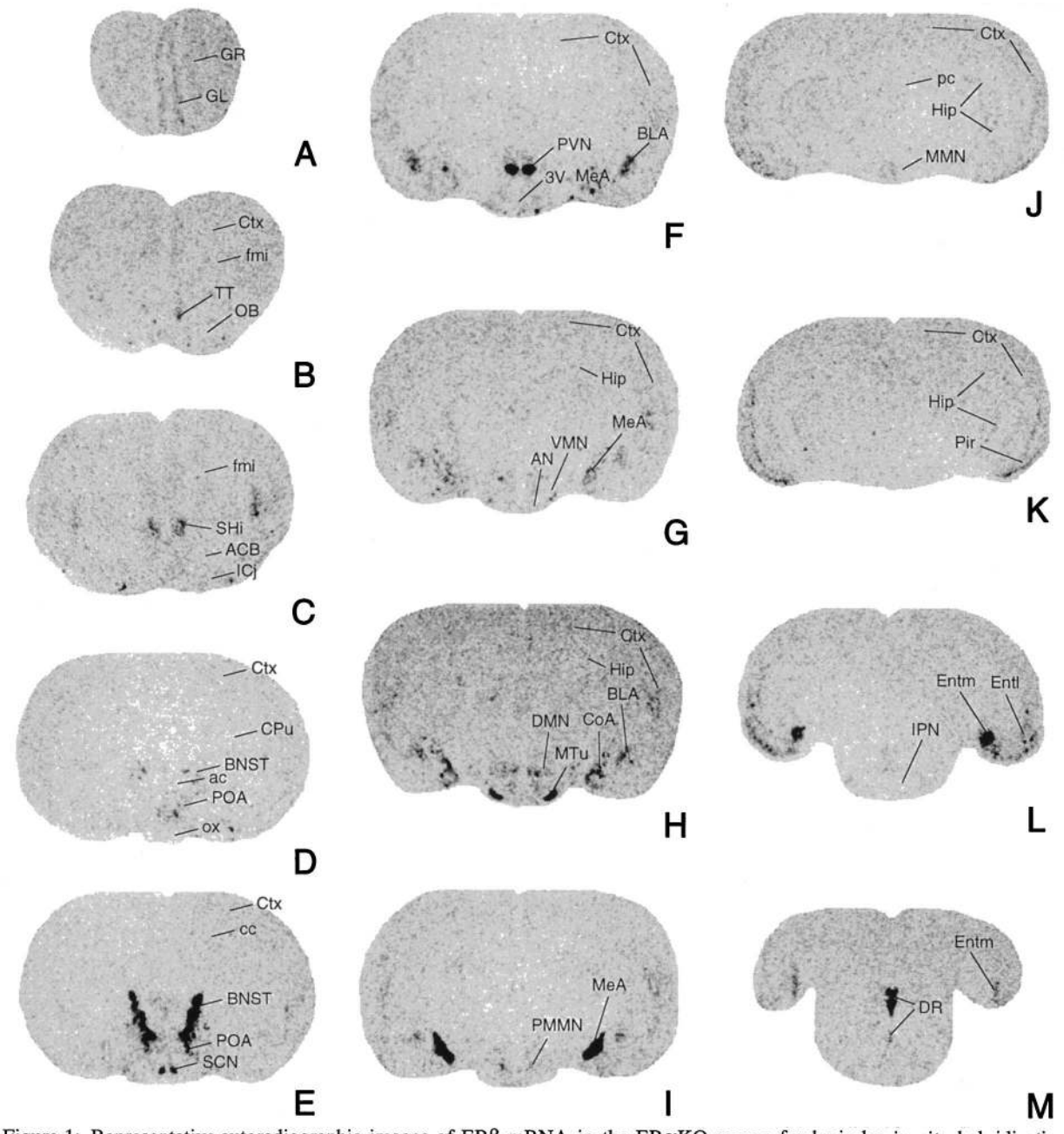

Figure 1: Representative autoradiographic images of ERβ mRNA in the ERαKO mouse forebrain by *in situ* hybridization (A-M). Note the concentration of ERβ mRNA in the bed nucleus ot the stria terminalis, suprachiasmatic nucleus, paraventricular nucleus, medial tuberal nucleus, medial amygdala and the medial portion of the entorhinal cortex. Additional hybridization signal was also seen in the preoptic area, dorsomedial nucleus, basolateral and cortical nuclei of the amygdala, cerebral and piriform cortex and hippocampus. Abbreviations: ac, anterior commissure; ACB, accumbens nucleus, AN, arcuate nucleus; BLA, basolateral amygdaloid nucleus; BNST; bed nucleus of the stria terminalis; cc, corpus collosum; CoA, cortical nucleus of the amygdala; CPu, caudate putamen; Ctx, cerebral cortex; DMN, dorsomedial hypothalamic nucleus; DR, dorsal raphe; Entl, entorhinal cortex lateral portion; Entm, entorhinal cortex medial portion; fmi, forceps minor of corpus collosum; GL, glomerular layer of the olfactory bulb; GR, granular layer of the olfactory bulb; Hip, hippocampus; ICj, islands of Calleja; IPN, interpeduncular nucleus; MeA, medial nucleus of the amygdala; MMN, medial mammillary nucleus; MTu, medial tuberal nucleus; OB, olfactory bulb; ox, optic chiasm; pc, posterior commissure; Pir, piriform cortex; PMMN, premammillary nucleus; POA, medial preoptic area; PVN, paraventricular hypothalamic nucleus; SCN, suprachiasmatic nucleus; SHi, septohippocampal nucleus; TT, tenia tecta; VMN, ventromedial hypothalamic nucleus; 3V, third ventricle.

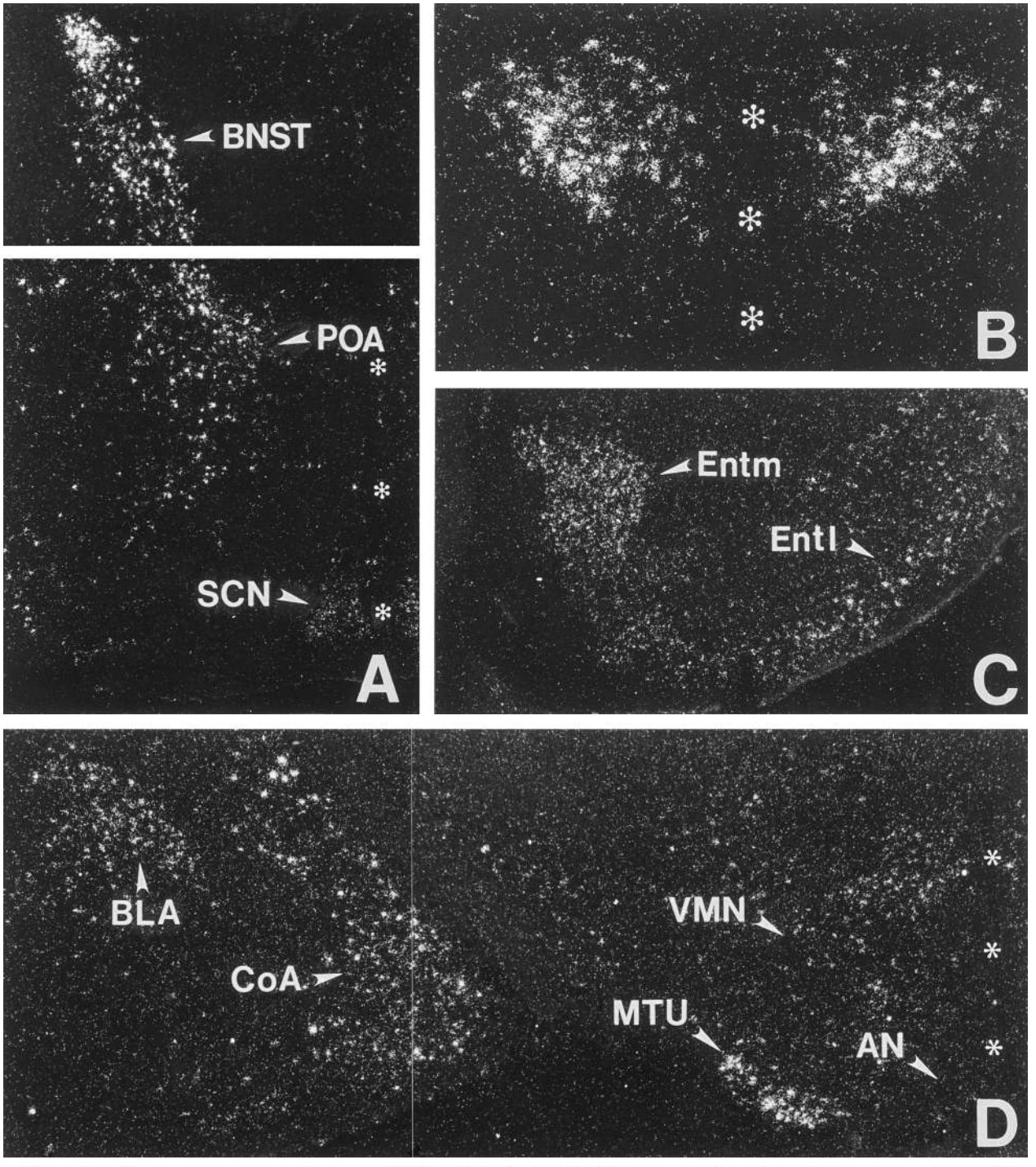

Figure 2: Representative autoradiograms of ERβ mRNA in the ERαKO mouse bed nucleus of the stria terminalis (A), medial preoptic area (A) suprachiasmatic nucleus (A), paraventricular nucleus (B), entorhinal cortex (C), amygdala (D) and medial tuberal nucleus (D) by *in situ* hybridization. Note that ERβ mRNA-containing perikarya are sparse or absent in the ventromedial and arcuate nuclei of the hypothalamus (D). Abbreviations: AN, arcuate nucleus; BLA, basolateral nucleus of the amygdala; BNST; bed nucleus of the stria terminalis; CoA, cortical nucleus of the amygdala; Entl, entorhinal cortex lateral portion; Entm, entorhinal cortex medial portion; MTu, medial tuberal nucleus; POA, medial preoptic area; SCN, suprachiasmatic nucleus; VMN, ventromedial hypothalamic nucleus. Asterisks indicate third ventricle.

Slides were then dehydrated with a graded series of alcohol: ammonium acetate, apposed to X-ray film and dipped in NTB2 nuclear emulsion. The slides were exposed for 2-4 weeks, photographically processed, stained in cresyl violet and coverslipped. Details concerning the *in situ* hybridization method have been reported previously (9).

Evaluation: Film autoradiographic images were used to evaluate the distribution of hybridization signal in the ERαKO mouse forebrain regions. The autoradiograms were digitized with a computer assisted image analysis system (C-Imaging Inc., Pittsburgh, PA), processed for contrast enhancement, imported into the Canvas illustrator program (Deneba Systems, Miami, Fl) and the image excised from the background and arranged into plates.

Results and Discussion

The results of these studies have demonstrated that ERβ mRNA is expressed in specific regions of the ERαKO mouse forebrain. In the hypothalamus, specific hybridization signal was seen in the medial preoptic area (Figs. 1D-E, 2A), suprachiasmatic nucleus (Figs. 1E, 2A), paraventricular nucleus (Figs. 1F, 2B), dorsomedial nucleus (Fig. 1H), medial tuberal nucleus (Figs. 1H, 2D), and the premammillary nuclei (Fig. 1I). Additional labeled perikarya were also detected in the glomerular layer of the olfactory bulb (Fig. 1A); tenia tecta (Fig. 1B); anterior septum; bed nucleus of the stria terminalis (Figs. 1D-E, 2A); medial, basolateral and cortical amygdaloid nuclei (Figs. 1F-I, 2D); cerebral and entorhinal cortex (Figs. 1I-M, 2C); septohippocampal nucleus (Fig. 1C); the hippocampus (Figs. 1J-K) and dorsal raphe (Fig. 1M).

Previous studies have detected ERβ mRNA and protein in many rat brain regions including the olfactory bulb, medial preoptic area, bed nucleus of the stria terminalis, supraoptic nucleus, paraventricular nucleus, arcuate nucleus, medial tuberal nucleus, amygdala and throughout the rostral-caudal extent of the cortex and hippocampus (5-7). The present findings in the ERαKO mouse brain are in good agreement with these studies, although some differences were observed. The most notable difference was the weak hybridization signal in the ERαKO mouse supraoptic nucleus, an area where ERβ mRNA was abundant in rat (5,6). The expression of ERβ mRNA was also attenuated in the anterior preoptic area, arcuate nucleus, ventral tegmental area and rostral levels of the cortex. In contrast, ERβ mRNA was abundant in the suprachiasmatic nucleus, basolateral amygdaloid nucleus and the medial aspect of the entorhinal cortex, areas that are weak or absent in rat brain (5,6). The differences between the expression of ERβ mRNA in the rat and ERαKO mouse brain appear to be due to species differences, since a comparable distribution of ERβ mRNA was detected in the wild type mouse brain (Shughrue and Merchenthaler, unpublished observations).

The finding that ERβ mRNA is expressed in the ERαKO mouse brain, suggests that estrogen may still regulate the expression of genes in the absence of ERα. This is supported by the recent observation that (i) radiolabeled estrogen is concentrated in the cell nucleus of neurons in several regions of the ERαKO mouse brain including the medial preoptic nucleus (3) and (ii) estrogen regulates the expression of progesterone receptor mRNA in this same brain region (3). Together, these observations and the present finding that the ERαKO mouse medial preoptic nucleus also expresses ERβ mRNA, suggest that ERα may not be required for the normal development and maintenance of certain neuronal functions in the brain. However, the finding that ERαKO mice have deficiencies in reproductive and maternal behavior (2) demonstrates that ERα is required for the normal development of some neuronal mechanisms. These deficiencies are likely due to the loss of ERα in regions such as the ventromedial nucleus, an area involved in reproductive behavior, and absence of ERβ to compensate for this loss (Figs. 1G-H, 2D). Future studies are clearly needed to resolve the importance of each ER and the interaction of both receptors in the brain.

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