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DIFFERENTIAL DISTRIBUTION OF ESTROGEN RECEPTOR (ER)-α AND ER-β IN THE MIDBRAIN RAPHE NUCLEI AND PERIAQUEDUCTAL GRAY IN MALE MOUSE: PREDOMINANT ROLE OF ER-β IN MIDBRAIN SEROTONERGIC SYSTEMS

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Abstract—We examined the distribution of estrogen receptor (ER)-α and ER-β immunoreactive (ir) cells in the dorsal (DRN) and median/paramedian (MPRN) raphe nuclei in male mice. ER-α ir neurons were scattered across the three subdivisions (ventral, dorsal, and lateral) of the DRN and the MPRN. Robust ER-β ir cells were observed throughout the raphe nuclei, and were particularly abundant in the ventral and dorsal subdivisions of the DRN. Using dual-label immunocytochemistry for ER-α or ER-β with tryptophan hydroxylase (TPH), the rate-limiting enzyme for 5-hydroxytryptamine (5-HT) synthesis, over 90% of ER-β ir cells exhibited TPH-ir in all DRN subdivisions, whereas only 23% of ER-α ir cells contained TPH. Comparisons of ER-α knockout (αERKO) as well as ER-β knockout (βERKO) mice with their respective wild-type (WT) littermates revealed that gene disruption of either ER-α or ER-β did not affect the other ER subtype expression in the raphe nuclei. *In situ* hybridization histochemistry revealed that there was a small but statistically significant decrease in TPH mRNA expression in the ventral DRN subdivision in βERKO mice compared with βWT mice, whereas TPH mRNA levels were not affected in αERKO mice. These findings support a hypothesis that ER-β activation may contribute to the estrogenic regulation of neuroendocrine and behavioral functions, in part, by acting directly on 5-HT neurons in the raphe nuclei in male mice.

Key words: serotonin, aggression, tryptophan hydroxylase, sexual behavior, knockout mouse, immunocytochemistry.

It is well established that estradiol (E_2), an aromatized metabolite of testosterone, plays a crucial role in neuroendocrine processes and reproduction-related behaviors in males (Meisel and Sachs, 1994; Ogawa et al., 2002; Olsen, 1992; Simon and Whalen, 1987). These estrogenic actions are mediated through at least two subtypes of estrogen receptors (ERs), ER-α (Green et al., 1986) and ER-β (Kuiper et al., 1996; Tremblay et al., 1997), which have similar binding affinities for the endogenous ligand, E_2. Both ERs act as ligand-activated transcription factors that regulate the transcription of target genes. A series of behavioral studies using single (αERKO and βERKO) and double (αβERKO) knockout mice for ER genes have revealed that activation of ER-α and ER-β is indeed involved in the regulation of sexual and aggressive behaviors in male mice (Imwalle et al., 2002; Nomura et al., 2002; Ogawa et al., 1997, 1998, 1999, 2000, 2002; Rissman et al., 1999; Scordalakes and Rissman, 2003; Wersinger and Rissman, 2000; Wersinger et al., 1997). These studies also collectively suggest that ER-α and ER-β might play different roles for the control of male sexual and aggressive behaviors.

Neuroanatomical studies have revealed that although both types of ERs are widely distributed in brain regions responsible for sociosexual behaviors, such as the hypothalamus, limbic system, and midbrain, their distribution patterns are not completely overlapping (Laflamme et al., 1998; Mitra et al., 2003; Shughrue et al., 1997a). Among them, the ascending midbrain raphe nuclei, consisting of the dorsal (DRN) and median/paramedian (MPRN) raphe, and the midbrain periaqueductal gray (PAG) are particularly interesting. It is well established that these midbrain regions are involved in the control of male sexual and aggressive behaviors based on lesion, electrical stimulation, and anatomical tracing studies (File and Deakin, 1980; Murphy and Hoffman, 2001; Vergnes et al., 1988; Yamamoto and Ueki, 1977). Several studies have shown that both DRN and PAG exhibit ER protein and mRNA in a number of species including mice (Alves et al., 1998, 2000; Lu et al., 2001; Mitra et al., 2003; Murphy et al., 1999; Nomura et al., 2003; Shughrue and Merchenthaler, 2001; Turcotte and Blaustein, 1993; VanderHorst et al., 1998). These studies have consistently shown that ER-α immunoreactive (ir) cells are localized in both the raphe nucleus

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Abbreviations: ANOVA, analysis of variance; BNST, bed nucleus of stria terminalis; DAB, 3,3′-diaminobenzidine; DRN, dorsal raphe; ER, estrogen receptor; ir, immunoreactive; MPOA, medial preoptic area; MPRN, median/paramedian raphe; nPGi, nucleus paragigantocellularis; PAG, periaqueductal gray; PB, phosphate buffer; PBS, phosphate buffer saline; PR, progesterone receptor; PVN, paraventricular nucleus; TPH, tryptophan hydroxylase; 5-HT, 5-hydroxytryptamine, serotonin.

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and the PAG, whereas ER-β ir as well as ER-β mRNA are expressed in the raphe nuclei, but not in the PAG. However, most of these anatomical studies are performed in females and the detailed distribution of both ER subtypes in the male raphe nuclei and PAG along the rostro-caudal extent or in the different subdivisions of the DRN has not yet been described. Furthermore, it is not known whether gene disruption of one ER may affect the expression of the other subtype in the DRN and PAG, as found in hypothalamic brain regions (Nomura et al., 2003; Temple et al., 2001). Therefore, in the first part of the present study, we examined the distribution of ER-α or ER-β ir cells in three subdivisions (i.e. dorsal, ventral, and lateral) of the DRN, the MPRN, and the PAG along the entire rostro-caudal extent in gonadally intact male mice. In order to explore the possible effects of either ER-α or ER-β gene disruption on the expression of the other subtype of ER, we utilized αERKO and βERKO mice and compared results with their respective WT siblings.

One of the targets of E_2 action in the DRN for the control of sociosexual behaviors is serotonin (5-hydroxytryptamine; 5-HT) synthesizing neurons. It has been shown that E_2 directly modulates the midbrain serotonergic system including increasing the expression of tryptophan hydroxylase (TPH), the rate-limiting enzyme for 5-HT synthesis, 5-HT transporter (SERT), and the autoreceptor, 5-HT_{1A} (Lu et al., 1999; McQueen et al., 1997, 1999; Pecins-Thompson and Bethea, 1999; Pecins-Thompson et al., 1996, 1998). Recent studies in rats and macaques have also shown that TPH co-localizes with ER-β in the DRN (Gundlah et al., 2001; Lu et al., 2001). These findings suggest that behavioral modifications in αERKO, βERKO, and αβERKO mice might be partly due to altered estrogenic actions on the serotonergic system at the level of the midbrain raphe nuclei. However, the degree of co-localization of TPH with ER-α and that with ER-β in different subdivisions of the DRN have not been directly compared in the same animals. Furthermore, it is still unknown how deletion of ER-α or ER-β genes may affect the TPH expression in the raphe nuclei. In the second part of the study, therefore, we compared the numbers of cells co-expressing TPH and ER-α ir or those co-expressing TPH and ER-β ir in each subdivision of the DRN in WT control mice, using dual-label immunocytochemistry. In addition, we examined the levels of TPH mRNA expression in the three subdivisions of the DRN and the MPRN in αERKO and βERKO mice and compared this expression to those of respective WT control mice.

EXPERIMENTAL PROCEDURES

Mice

Gonadally intact adult male αERKO (Lubahn et al., 1993) and βERKO (Krege et al., 1998) mice and their respective WT (αWT and βWT) littermates at age 9–14 weeks were used. They were obtained from the αERKO and the βERKO breeding colonies maintained at the Rockefeller University by mating heterozygous male and female mice. Original breeding pairs (mixed background of C57BL/6J and 129) were obtained from the National Institute of Environmental Health Sciences. Mice were group-housed (four to five mice/cage) in plastic cages (30×20×12 cm) at constant temperature (22 °C) on a 12-h light/dark cycle. Food and water were available *ad libitum* throughout the experiment. All procedures were approved by the IACUC of the Rockefeller University and named guidelines on the ethical use of animals. We minimized the number of animals used and their suffering during the experiments.

Immunohistochemistry

Total of 24 mice (n=6/genotype) were deeply anesthetized using sodium pentobarbital (75 mg/kg; ip) and perfused via the left ventricle with phosphate buffer saline (PBS) containing 0.1% heparin, pH 7.4, followed by 2% acrolein and 2.5% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. After postfixation, the brains were equilibrated in PB containing 30% sucrose for 48 h at 4 °C. The brain tissues were frozen with powdered dry ice, coronally sectioned at 40 μm on a freezing microtome, and stored in cryoprotectant (30% glycerol, 30% ethylene glycol in 0.1 M PB) at −20 °C until they were used.

Free floating sections were thoroughly washed in 0.1 M PBS and then were washed in 0.1% sodium borohydride ($NaBH_4$). After several washes in PBS, endogenous peroxidase activity was blocked by washing tissue in 0.1% hydrogen peroxide in PBS for 20 min. Sections were washed several times in PBS and then blocked with either 2% normal rabbit (for detection of ER-α) or goat serum (for ER-β). Sections were then incubated in a rat monoclonal ER-α antibody, H222 (1:1000; a gift of Dr. G. Greene, University of Chicago) or a rabbit polyclonal ER-β antibody, Z8P (1:10,000; Zymed Laboratory Inc., CA, USA; Shughrue and Merchenthaler, 2001) in 0.1 M PBS containing 0.5% Triton X-100 and 2% normal rabbit (for ER-α) or goat serum (for ER-β) for 48 h at 4 °C. The sections were further incubated with a 1:200 dilution of the biotinylated anti-rat (for ER-α) or anti-rabbit (for ER-β) secondary antibody (Vector Laboratories) in PBS containing 0.5% Triton X-100 and 2% normal serum for 120 min at room temperature. The sections were finally incubated with avidin–biotin peroxidase complex (Vectastain ABC Elite Kit; Vector Laboratories) in PBS for 90 min. Following several washes in PBS, the sections were developed with 0.03% 3,3′-diaminobenzidine (DAB) containing 0.15% nickel ammonium sulfate and 0.03% hydrogen peroxide in PBS for 3 min. Sections were then mounted on gelatin-coated slides and air dried. The slides were dehydrated through an ascending alcohol series, cleared with xylene, and coverslipped with Permount (Fisher Scientific).

Dual-label immunocytochemistry

A separate set of βWT brain samples (n=4) was prepared and the free-floating sections (30 μm thickness) were processed for immunocytochemistry as described above, except 0.3% skim milk was used instead of normal serum. Sections were first incubated in an ER-α antibody, H222 (1:1000) or an ER-β antibody, Z8P (1:10,000) for 2 days at 4 °C. The peroxide complex was visualized with 0.02% DAB containing 2.5% nickel sulfate and 0.01% hydrogen peroxide in a 0.175 M sodium acetate buffer for 2 min, as a dark purple punctate stain. After being washed in PBS containing 3% skim milk overnight at 4 °C, the sections were then incubated in a sheep polyclonal TPH antibody (1:8000; Chemicon International Inc.) for 2 days at 4 °C. The peroxidase complex in the section was visualized with 0.05% DAB containing 0.1% hydrogen peroxide for 2 min, as a light brown cytoplasmic stain.

In situ hybridization histochemistry

Total of 24 mice (n=6/genotype) were decapitated and the brains were rapidly removed, frozen on powdered dry ice and stored at −80 °C until they were used. Frozen section were cut at 12 μm on a cryostat and mounted onto coated slides (Fisher Scientific). The ER-β probe was generated from a 2100-bp fragment of the mouse