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Distribution of estrogen receptor beta containing cells in the brains of bacterial artificial chromosome transgenic mice

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Abstract

In the brain, estrogen receptor β (ER β) plays important roles in autonomic functions, stress reactivity and learning and memory processes. However, understanding the function of ER β has been restricted by the limited availability of specific antisera, by difficulties discriminating the discrete localization of ER β -immunoreactivity (ir) at the light microscopic level in many brain regions and the identification of ER β -containing neurons in neurophysiological and molecular studies. Here, we demonstrate that a *Esr2* bacterial artificial chromosome (BAC) transgenic mouse line that expresses ER β identified by enhanced green fluorescent protein (EGFP) overcomes these shortcomings. Throughout the brain, ER β -EGFP was detected in the nuclei and cytoplasm of cells, the majority of which resembled neurons. EGFP often extended into dendritic processes and could be identified either natively or following intensification of EGFP using immunolabeling. The distribution of ER β -EGFP cells in brain closely corresponded to that reported for ER β protein and mRNA. In particular, ER β -EGFP cells were found in autonomic brain regions (i.e., hypothalamic paraventricular nucleus, rostral ventrolateral medulla and nucleus of the solitary tract), in regions associated with anxiety and stress behaviors (i.e., bed nucleus of the stria terminalis, amygdala, periaqueductal gray, raphe and parabrachial nuclei) and in regions involved in learning and memory processes (i.e., basal forebrain, cerebral cortex and hippocampus). Additionally, dual label light and electron microscopic studies in select brain areas demonstrate that cell containing ER β -EGFP colocalize with both nuclear and extranuclear ER β -immunoreactivity. These findings support the utility of *Esr2* BAC transgenic reporter mice for future studies understanding the role of ER β in CNS function.

Keywords

autonomic nuclei; amygdala; raphe nuclei; hippocampus; cerebral cortex; hypothalamus

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1. INTRODUCTION

Estrogen receptor β (ER β) plays important roles in many functions throughout the central nervous system (CNS). In particular, ER β is involved in autonomic functions (Bingham et al., 2006; Laflamme et al., 1998; Wang et al., 2006b). ER β gene function is important for the estrogen regulation of anxiety and stress reactivity (Tomihara et al., 2009; Imwalle et al., 2005; Walf and Frye, 2007; Walf et al., 2008b). Moreover, ER β is involved in learning and memory processes (Liu et al., 2008). Previous anatomical studies have shown that ER β -immunoreactivity (ir) and mRNA are expressed in brain regions that are known to be importantly involved in autonomic regulation, stress reactivity and mnemonic processes, including the paraventricular nucleus (PVN) of the hypothalamus, amygdala, the bed nucleus of the stria terminalis, hippocampus, and the raphe nuclei (Merchenthaler et al., 2004; Shughrue et al., 1997; Shughrue and Merchenthaler, 2001; Mitra et al., 2003).

Characterizing the phenotype and understanding the function of ER β -containing cells in the CNS has been hampered by several factors. First, many of antibodies to ER β lack specificity or may not recognize all the isoforms of ER β (Snyder et al., 2010) (also see discussion in (Milner et al., 2005)). Additionally, two of the specific antibodies to ER β (i.e., those from Merck and from Zymed) which we used in our previous studies (Herrick et al., 2006; Milner et al., 2005; Mitterling et al., 2010) are no longer available. Second, in addition to nuclei, ER β is found at extranuclear locations in several brain regions (Milner et al., 2005; Wang et al., 2006b; McEwen and Milner, 2007; Milner et al., 2003; Mitra et al., 2003). In particular, our electron microscopic immunocytochemical studies have shown that extranuclear ER β -ir is found in discrete patches adjacent to membranes or in small cellular profiles which cannot be discerned at the light microscopic level (Herrick et al., 2006; Milner et al., 2005; Wang et al., 2006b). Third, detection of ER β -ir, particularly extranuclear ER β , is optimal in brain tissue that is perfusion fixed with paraformaldehyde containing acrolein (Herrick et al., 2006; Milner et al., 2005; Mitterling et al., 2010; Mitra et al., 2003). Unfortunately, acrolein can autofluoresce making it difficult to visualize low levels of nuclear or extranuclear ER β ir with immunofluorescence. Fourth, detergents and other tissue penetration enhancing methods severely reduce the visualization of extranuclear ER β -ir in both light and electron microscopic experiments resulting in false negatives in dual labeling studies [e.g., (Herrick et al., 2006; Torres-Reveron et al., 2009)]. Fifth, understanding the function of ER β has been limited due to lack of identification of ER β -containing neurons in neurophysiological and molecular studies dealing with co-expression.

In this study, we describe an Esr2 (ER β) bacterial artificial chromosome (BAC) transgenic reporter mouse line obtained from the Gene Expression Nervous System Atlas (GENSAT) project (Gong et al., 2003) that overcomes these shortcomings. Throughout the brain, enhanced green fluorescent protein (EGFP) indicative of ER β was detected throughout cells allowing them to be morphologically defined as neurons. These EGFP cells could be identified either natively supporting their utility for electrophysiological and molecular studies or in acrolein and paraformaldehyde fixed tissues supporting their utility in electron microscopic studies. The distribution of ER β -EGFP cells throughout the brain was consistent with that shown previously for ER β immunoreactivity and mRNA. Moreover, ER β -EGFP was colocalized with both nuclear and extranuclear ER β -immunoreactivity supporting the fidelity of the Esr2 BAC transgenic reporter mouse line for future studies.

2. RESULTS

2.1 Description of Esr2 (ER β) BAC transgenic mouse line

BAC-based Esr2-EGFP mice, currently on a mixed FVB/N and C57BL/6 background, were obtained from the GENSAT Project (www.gensat.org) at the Rockefeller University (Gong

et al., 2003). The mice were generated by inserting an EGFP cassette directly upstream of the coding sequence of ER β into a BAC using a homologous recombination (see Experimental Procedure 4.1 for details). The native copy of the *Esr2* gene on chromosome 12 of the mouse remained untouched and thus cells with EGFP did not have any more or less ER β expression than normal.

To assess the fidelity of the *Esr2*- BAC transgenic reporter mice, we first assessed the cellular distribution of EGFP containing cells in the brains of females and males to determine how it compares to that reported previously for ER β protein and message (Mitra et al., 2003; Shughrue et al., 1997; Shughrue and Merchenthaler, 2001; Merchenthaler et al., 2004). Next, at the light and electron microscopic levels, we dually labeled sections for EGFP and ER β -ir from brain regions known to contain abundant nuclear and/or extranuclear ER β .

2.2 Morphology of EGFP cells in the *Esr2* (ER β) BAC transgenic mouse brain

EGFP cells were examined in the brains of both female and male *Esr2* BAC transgenic reporter mice identified either with native fluorescence or enhanced with immunocytochemistry. At the light microscopic level, EGFP was detected in the nuclei and cytoplasm of cells (Fig. 1A-D). The majority of EGFP labeling in *Esr2* BAC mice (i.e., ER β -EGFP) was detected in cells with the morphological characteristics of neurons (Cajal, 1995) that ranged in size from 10 – 25 μ m. ER β -EGFP often filled proximal dendrites. For example, EGFP reaction product filled cells in many brain areas allowing for the distinction of fusiform or bipolar shapes (Fig. 1A, C-D). Moreover, in layer 5 of the cerebral cortex ER β -EGFP was in the apical dendrites of pyramidal cells that often extended to the more superficial layers (Fig. 1B). In general, the distribution and morphology of ER β -EGFP cells were similar in female and male mice. Furthermore, the intensity of ER β -EGFP appeared similar in females and males. However, quantitative analysis revealed that female *Esr2* BAC transgenic mice had more ER β -EGFP cells than males in some brain regions (Table 1).

To determine if *Esr2* BAC transgenic mice brains were suitable for electrophysiological and molecular studies, we examined native EGFP labeled cells in the PVN of the hypothalamus, which is abundant with ER β containing cells (Hrabovszky et al., 1998; Bingham et al., 2006; Laflamme et al., 1998). For this, 200 μ m-thick coronal sections suitable for electrophysiological studies (see Experimental Protocol sec. 4.2.5) were prepared from the PVN region of the hypothalamus of both homo- and heterozygous transgenic ER β -EGFP mice. When viewed under bright field, the fornix, optic chiasm and third ventricle were easily seen and used as landmarks for identifying the PVN (Fig. 1E). When viewed under fluorescence, native fluorescence for EGFP was readily detectable in the PVN of both male and female (Fig. 1F). Additional dual labeling with retrogradely transported Texas red beads revealed that the native EGFP fluorescence was clearly distinguishable from the Texas red beads (Fig. 1G). We next prepared isolated cells from the PVN region using protocols suitable for patch clamp recording and single cell reverse transcriptase polymerase chain reaction (RT-PCR) (Wang et al., 2006b). We found that isolated EGFP-containing cells from the PVN of both female and male *Esr2*-EGFP BAC transgenic mice maintained intense native EGFP labeling (Fig. 1H, I). Thus, these findings demonstrate the utility of the *Esr2* BAC transgenic mice in electrophysiological and molecular studies.

2.3 Distribution of EGFP cells in the *Esr2* BAC transgenic mouse brain

We next determined the distribution of EGFP containing cells in the brains of *Esr2* BAC transgenic mice to compare with what has been shown previously for ER β -ir and mRNA in the brains of rats and mice (Mitra et al., 2003; Shughrue et al., 1997; Shughrue and Merchenthaler, 2001; Merchenthaler et al., 2004). For the anatomical studies presented

below, EGFP was localized in *Esr2* BAC transgenic mice using peroxidase immunocytochemical methods to increase the signal and cellular resolution of EGFP as well as yield a permanent reaction product that was visible under the light microscope. The distribution pattern was identical in females and males. However, as noted above, females had more EGFP cells than males in some brain regions. The maps presented in Figure 2 are a composite of the distribution of EGFP cells made from examining the brains of 4 female (all in diestrus, i.e., the low estrogen stage of the estrous cycle) and 4 male *Esr2* BAC transgenic mice. The maps present relative qualitative comparisons of the numbers of cells in different brain regions.

2.3.1 Forebrain—ER β -EGFP cells were found in the olfactory nucleus and affiliated brain regions. In particular, ER β -EGFP cells were dispersed throughout all subdivisions of the anterior olfactory nucleus (Fig. 2A) and the endopiriform nucleus and the piriform cortex (Fig. 2A-F). Dense clusters of ER β -EGFP cells were consistently observed in the tenia tecta, olfactory tubercle and insula of Calleja (Fig. 2B).

The basal forebrain nuclei contained numerous ER β -EGFP cells. Many ER β -EGFP cells were found in the entire rostrocaudal extent of the dorsal and lateral portions of the lateral septal nuclei (Figs. 2B-C; 3A). ER β -EGFP cells were distributed throughout the medial septum and diagonal band of Broca (Fig. 2B) and nucleus basalis. Scattered ER β -EGFP cells were found in the core and shell of the nucleus accumbens (Figs. 1B, 2A), all subdivisions of the bed nucleus of the stria terminalis (Fig. 1C), medial preoptic area (Fig. 1C) and substantia innominata (Fig. 2C). Notably, almost no ER β -EGFP cells were seen in the caudate putamen and globus pallidus (Fig. 2B-D).

2.3.2 Thalamus, amygdala and hypothalamus—Only a few regions in the thalamus contained ER β -EGFP cells. In the rostral regions of the thalamus, a dense cluster of ER β -EGFP cells was found in the caudolateral habenula (Figs. 2E, 3C). A few scattered ER β -EGFP cells were found in the anterodorsal, anteroventral and laterodorsal nuclei of the thalamus (Fig. 2D). A stream of ER β -EGFP cells was detected in the reticular thalamic nucleus and neighboring ventral anterior nucleus of the thalamus and zona incerta (Fig. 2D). More caudally, the ventral subregions of the lateral geniculate (Fig. 2E) and the medial geniculate (Fig. 2G) contained small clusters of ER β -EGFP cells. Additionally, a few scattered ER β -EGFP cells were observed in the ventral tegmental nucleus (VTA), red nucleus and substantia nigra, especially the pars compacta (Fig. 2G).

ER β -EGFP cells were detected in many of the nuclei within the amygdala complex. Numerous ER β -EGFP cells were in the medial nucleus of the amygdala, particularly in the caudal portion (Fig. 8B) and in the lateral and basolateral nucleus of the amygdala (Figs 2D; 2B). The central nucleus of the amygdala was noticeably devoid of ER β -EGFP cells (Fig. 2B). Moreover, few ER β -EGFP cells were found in the basomedial nucleus of the amygdala (Fig. 2D) and the posterior lateral and medial cortical nucleus of the amygdala (Fig. 2 E & F).

In the hypothalamus, ER β -EGFP cells were densely packed in the PVN (Figs. 2D; 4A). When viewed at higher magnification, dendritic processes of ER β -EGFP neurons could be distinguished (Fig. 4A, inset). The distribution of ER β -EGFP cells in the PVN resembles that of ER β immunoreactive cells in both mice and rats (Fig. 4C,D). However, in mice ER β -EGFP cells were more diffuse compared to rats. Thus, to elucidate the location of EGFP cells with PVN subregions, sections through the PVN were dually labeled with vasopressin (AVP), a marker of magnocellular neurons (Coleman et al., 2009). Most ER β -EGFP cells were medial to and intermixed with AVP-labeled neurons suggesting that most ER β -EGFP cells were parvocellular neurons. In addition to the PVN, several ER β -EGFP cells were

detected in the arcuate nucleus, dorsomedial and ventromedial hypothalamic nucleus, periventricular nucleus of the hypothalamus, the suprachiasmatic nucleus (not shown), the caudal supraoptic nucleus (Fig. 2C,D) and dorsal region of the periaqueductal gray area (Fig. 2G). A few scattered ER β -EGFP cells were observed in the medial preoptic area, the anterior hypothalamic nucleus, lateral hypothalamic area (Fig. 2D,E) and the medial and lateral tuberal nucleus (not shown). Both the medial and lateral regions of the supramammillary nucleus contained several ER β -EGFP cells (Fig. 2F).

2.3.3 Cerebral and cerebellar cortices and hippocampus—Numerous ER β -EGFP cells were found throughout the rostrocaudal extent of all cerebral cortical subdivisions (Fig. 2A-H; 5A). Most ER β -EGFP cells were located in layer 5 and in the deep aspects of layer 6, immediately dorsal to the corpus callosum (Fig. 5A,B). Most of the ER β -EGFP cells in layer 5 had the morphology of pyramidal cells and had long dendritic processes that extended dorsally into layer 4 (Figs. 1B; 4B). Many small ER β -EGFP cells were detected in the principal cell layers of the anterior cingulate and retrosplenial cortices, especially the ventral aspects (Fig. 2B-F; 5A).

ER β -EGFP cells were detected throughout the hippocampal formation, however, the density of labeled cells varied by subregion (Fig. 2E-G). The dorsal and ventral subiculum contained a dense accumulation of ER β -EGFP cells (Fig. 5A; Table 1). Scattered intensely labeled ER β -EGFP cells were found dorsal and ventral to the pyramidal cell layer in both CA1 and CA3 (Fig. 5C). Many intensely labeled ER β -EGFP cells were seen in the caudal aspects of the hilus of the dentate gyrus (Figs. 2D; 5D). Based on location and morphology, the intensely labeled ER β -EGFP cells in all three regions were likely interneurons (Freund and Buzsáki, 1996). In addition to these intensely labeled cells, less intense ER β -EGFP cells were found in the pyramidal cell layer, especially in the CA1 region (Fig. 5C) and in the subgranular region of the dentate gyrus (Fig. 5D). Moreover, lightly labeled ER β -EGFP processes were seen throughout the other cell layers, particularly stratum lacunosum-moleculare (Fig. 5A,C). ER β -EGFP cells were sparsely distributed in layer 5 and 6 of the entorhinal cortex in a manner similar to that described above for the cerebral cortex (Fig. 2G,H).

In the cerebellar cortex, ER β -EGFP cells were mostly detected overlying the white matter tracts (Fig. 2I,J). Occasionally, scattered ER β -EGFP cells were observed in the ventral aspects of the purkinje cell layer, especially in the central lobe (Fig. 2I,J).

2.3.4 Pons, medulla and spinal cord—Within the pons, the majority of ER β -EGFP cells were detected in the rostrocaudal extent of the raphe nuclei, especially the dorsal subgroup (Fig. 2H; 6A). Consistent with previous studies (Alves et al., 1998), additional dual label studies demonstrated that many of the ER β -EGFP cells in the dorsal raphe contained 5-HT-ir (not shown). Scattered ER β -EGFP cells were in the midline regions of the superior colliculus, the periaqueductal gray, intercollicular nucleus, pontine reticular nucleus (Fig. 2H) and medial to the rubrospinal tract (not shown). More caudally, numerous ER β -EGFP cells were detected in all subregions of the lateral parabrachial nucleus (Figs. 2I, 6B), in the midline region of the pontine central gray, the caudal portion of the dorsal tegmental nucleus and in the principal sensory nucleus of the trigeminal nerve (Fig. 2H,I). A few ER β -EGFP cells were noted in the locus coeruleus, inferior colliculus, the dorsal and ventral cochlear nucleus and medial to the facial nucleus (VII) (Fig. 2H,I).

In the medulla, ER β -EGFP containing cells continued to be found in the raphe nuclei, especially the magnus subdivision (Fig. 2J). Additional dual label studies revealed that many of the ER β -EGFP cells in the dorsal magnus contained 5-HT-ir (Fig. 6C). Numerous ER β -EGFP cells were detected in the nucleus of the solitary tract, especially in the caudal medial

and central subdivisions (Figs. 2K; 7A). Within the rostral ventrolateral medulla, several scattered ER β -EGFP cells were detected (Figs. 2J; 7B). Scattered ER β -EGFP cells were seen in the nucleus prepositus, intermediate and medullary reticular nucleus and external cuneate nucleus (Fig. 2J,L). Several ER β -EGFP cells were present in both the interpolar and caudal parts of the spinal nucleus of the trigeminal nerve, especially medial to the spinal trigeminal tract (Fig. 2K,L).

In the spinal cord, numerous ER β -EGFP cells were seen in the substantia gelatinosa and lateral cervical nucleus, medial to the dorsal spinocerebellar tract (Fig. 2M). A few ER β -EGFP cells were detected dorsal to the central canal and in the ventral horn (Fig. 2M).

2.4 EGFP cells in the *Esr2* BAC reporter mouse contain ER β - immunoreactivity

Next, we confirmed the fidelity of EGFP in the *Esr2* BAC transgenic mouse by colocalizing EGFP with nuclear and extranuclear ER β -ir.

2.4.1 Light microscopic localization of EGFP and ER β -ir—Sections from brains of *Esr2* BAC transgenic mice fixed with 4% paraformaldehyde were dually labeled for GFP and ER β (Z8P antibody from Zymed, see Experimental Procedure sec. 4.2.2) in the presence of detergent to identify primarily nuclear ER β -ir. We focused on the PVN, medial amygdala, dorsal raphe and NTS as these brain regions are abundant in nuclei containing ER β (Merchenthaler et al., 2004; Shughrue et al., 1997; Shughrue and Merchenthaler, 2001; Mitra et al., 2003). In the PVN, medial amygdala and dorsal raphe, nuclei with ER β -ir were found in most cells that contained EGFP immunolabeling (Fig. 8A-C). In the caudal medial NTS, numerous EGFP-labeled cells contained nuclei with ER β -ir; additionally some EGFP cells contained diffuse ER β -ir in their cytoplasm (Fig. 8D).

The hippocampus was also examined since it contained both intensely and lightly labeled ER β -EGFP cells. We found that EGFP-containing interneurons contained nuclei with ER β -ir (Fig. 8E). However, pyramidal cells lightly labeled for EGFP contained diffuse ER β -ir in their cytoplasm (Fig. 8E). A similar cytoplasmic ER β -ir was detected in EGFP containing cells in several other brain regions. These include the lateral septum, basolateral amygdala and supraoptic nucleus (not shown).

2.4.2 Electron microscopic localization of EGFP and ER β -ir—Our previous studies (Herrick et al., 2006; Milner et al., 2005; Wang et al., 2006b; Milner et al., 2003) showed extranuclear ER β -ir in many brain regions. In most brain regions, extranuclear ER β -ir can only be detected by electron microscopy. Thus, sections from the brains of *Esr2* BAC transgenic mice fixed with acrolein and paraformaldehyde were dually labeled for GFP and ER β -ir and processed for electron microscopy (*Experimental Procedures* sec. 4.2.6). We focused on four regions known to contain extranuclear ER β . Before beginning the dual labeling electron microscopic studies, we determined if the ultrastructural labeling conditions for GFP in the PVN and hippocampus were compatible with ER β immunocytochemistry using immunoperoxidase alone.

PVN: In sections singly labeled for immunoperoxidase, ER β -EGFP was found in perikarya and dendrites within the PVN using labeling conditions compatible with ER β immunocytochemistry (Figs. 9A; 10A,B). When visualized with either peroxidase or silver-intensified gold (SIG) ER β -EGFP-ir was found throughout the perikarya and dendritic profiles (Figs. 9 & 10). Dual labeling studies revealed that extranuclear ER β -ir was adjacent to the plasma membranes of EGFP-containing perikarya (Fig. 9B). Moreover, unlike EGFP reaction product, ER β -ir was localized in discrete patches adjacent to mitochondria (Fig. 10C).

Medial amygdala: In sections dually labeled for EGFP and ER β -ir, numerous perikarya and dendritic processes contained both labels (Fig. 11). Interestingly, some EGFP perikarya contained only extranuclear ER β -ir whereas other cells contained only nuclear ER β -ir (Fig. 11A). EGFP SIG particles were usually distributed through the nucleus and cytoplasm of perikarya and dendrites whereas ER β -ir was often found in clusters. In the cytoplasm, ER β -ir was often near mitochondria (Fig. 11B).

Hippocampus: Consistent with our light microscopic observations, cellular profiles with of ER β -EGFP were less abundant in the hippocampal CA1 region than the PVN. In sections of the hippocampus singly labeled for GFP using peroxidase, ER β -EGFP reaction product was detected in the shafts of small dendrites where it was adjacent to the plasma membrane and rimmed mitochondria (Fig. 12A). Moreover, ER β -EGFP peroxidase reaction product was sometimes detected in unmyelinated axons (Fig. 12B). In dual labeled sections, EGFP SIG particles were dispersed throughout dendritic profiles whereas ER β -ir (as identified with peroxidase) was more discretely localized in dendritic spines (Fig. 12C,D).

Raphe magnus: In sections of the raphe magnus dually labeled for EGFP and ER β immunocytochemistry, reaction products for both antibodies were often found in the same perikarya and dendrites (Fig. 13). Like the other brain regions examined, EGFP SIG particles were found throughout the nucleus and cytoplasm of perikarya and dendrites whereas ER β -ir was more discrete. In the raphe magnus, nuclear and extranuclear ER β -ir was often found in the same EGFP perikaryon (Fig. 13).

3. DISCUSSION

These studies demonstrate that EGFP containing cells in BAC transgenic reporter mice have a cellular distribution throughout the brain that is similar to that reported previously for ER β mRNA and protein. Dual label light and electron microscopic studies demonstrate that EGFP cells colocalize with nuclear and extranuclear ER β -ir in many brain regions. The versatility and greater sensitivity of GFP for ER β in the *Esr2* BAC transgenic mouse will provide a useful tool for future molecular, physiological and anatomical investigations.

Methodological considerations

These studies support the feasibility of using *Esr2* BAC transgenic mice in physiological, molecular and anatomical studies. First, native EGFP fluorescence was visible at the light level in soma and proximal processes of cells allowing for their identification in fresh tissue slices and isolated neurons prepared for physiological and molecular studies. Moreover, the green signal of native EGFP was clearly distinguished from retrogradely transported Texas Red microbeads, allowing for the identification of projection specific ER β -EGFP neurons in physiological preparations. Second, the localization of immunoperoxidase or immunogold-silver reaction product for EGFP throughout dendritic processes is ideal for comparisons between treatment groups at the electron microscopic level of analysis. Our finding that silver intensified gold labeling for EGFP can be clearly distinguished from the peroxidase reaction product for ER β -ir in single sections prepared for electron microscopy further supports the technical feasibility of dual label ultrastructural studies. The observation that the peroxidase reaction product for EGFP often filled the entire dendritic profile is particularly important since we will less likely underestimate ER β -EGFP profiles in quantitative comparisons between experimental groups. Third, EGFP immunoreaction product can be detected in paraformaldehyde-fixed tissue with or without acrolein and in the presence or absence of detergent allowing ER β -containing cells to be identified under a greater variety of experimental conditions. This is particularly important since the identification of neurons containing extranuclear ER β has been limited by the labeling

conditions required for ER β antibodies [see (Herrick et al., 2006)]. Fourth, ER β -EGFP can be immunolabeled with a rabbit or chicken anti-GFP, allowing for greater versatility in dual light and electron microscopic studies since it can be combined with antibodies from a variety of species and can be identified with either fluorescence, peroxidase or immunogold labels.

The distribution of ER β -EGFP is consistent with immunocytochemical and *in situ* studies

The present study found the distribution of EGFP in the ESR2 BAC transgenic mouse to be consistent with previous reports from immunocytochemistry and *in situ* studies, with some variation in the degree of labeling. Our light and electron microscopic studies colocalizing nuclear and extranuclear ER β -ir in EGFP containing cells in many brain regions support the fidelity of the ESR2 BAC transgenic mice. The distribution of ER β -EGFP containing cells was similar in males and females. However, males had fewer ER β -EGFP containing cells in some brain regions.

In both female and male ESR2 BAC transgenic mice more robust populations of ER β -EGFP cells were found in the endopiriform nucleus, supramammillary nucleus, and the caudal hilus of the dentate gyrus, compared to previous immunocytochemical and *in situ* hybridization studies in mice and rats (Merchenthaler et al., 2004; Shughrue et al., 1997; Shughrue and Merchenthaler, 2001; Mitra et al., 2003). Most notably, the lateral septum was heavily populated with ER β -EGFP cells that previously have been shown to contain numerous ER β -immunoreactive cells but few cells with ER β mRNA (Li et al., 1997; Shughrue et al., 1997; Shughrue and Merchenthaler, 2001; Laflamme et al., 1998). While the general localization of the ER β is consistent across studies, it is worth noting that, in addition, ER β is consistently absent in certain brain areas including the striatum and many of the thalamic nuclei.

A number of factors may have contributed to the differences seen in labeling intensity. For one, the ESR2 BAC transgenic animals used in this study were of mixed FVB/N and C57BL/6 background, while previous immunocytochemical and *in situ* hybridization studies have used only C57BL/6 animals (Merchenthaler et al., 2004; Mitra et al., 2003). Additionally, ER β has 5 splice variants that may be differentially expressed throughout the brain (Price, Jr. et al., 2000; Weiser et al., 2008). Because the selectivity of ER β antibodies for these splice variants has not yet been determined, it is possible that previous immunocytochemistry studies underrepresented ER β localization due to an inability to detect certain isoforms of the receptor (Milner et al., 2005). The ESR2 BAC transgenic reporter mouse is created by inserting EGFP cassette directly upstream of the coding sequence of ER β into a BAC using a homologous recombination. Thus, this may allow for visualization of cells expressing low levels of ER β (Gong et al., 2003).

In all brain regions examined by electron microscopy, peroxidase and silver-intensified reaction product for GFP in the ESR2 BAC transgenic mice tended to fill somata and dendrites. This localization was consistent with that observed for GFP in other brain regions in several types of EGFP transgenic mice (Bullock et al., 2008; Justice et al., 2008; Lazarenko et al., 2009; Sierra et al., 2008). In comparison, immunoreactivity for ER β was more discrete, consistent with that observed in our previous studies (Herrick et al., 2006; Milner et al., 2005; Mitterling et al., 2010). Differences in ER β -EGFP labeling and ER β -ir likely reflect the increased sensitivity of BAC reporter gene vectors (Gong et al., 2003).

ER β -EGFP cells are present in several autonomic nuclei

Cells containing ER β -EGFP are found in several brain regions associated with autonomic functions, most notably the PVN of the hypothalamus and the RVLM. The PVN is involved

in the integration of neural control of sympathetic outflow (Benarroch, 2005; Sawchenko et al., 1996; de Wardener, 2001; Pyner and Coote, 1898). In particular, the PVN receives direct projections from the subfornical organ, an important regulator of humoral signals, as well as the NTS, the central site of the baroreceptor reflex (Bains and Ferguson, 1995; Hermes et al., 2006). In turn, the PVN directly projects to autonomic nuclei in the spinal cord and to a lesser extent, the spinal cord (Benarroch, 2005). In the PVN, ER β is importantly involved in the estrogen-mediated regulation of blood pressure (Gingerich and Krukoff, 2006). In rats, ER β containing PVN neurons that project to autonomic centers in the spinal cord and brainstem can be identified by corticotrophin releasing factor (CRF)-immunoreactivity (Laflamme et al., 1998; Bingham et al., 2006). However, in mice, the study of ER β -containing neurons has been hampered by the unavailability of specific ER β antibodies (Snyder et al., 2010) and the low detectability of CRF-ir in PVN neurons (Milner unpublished observations). Thus, the identification of the *Esr2* BAC transgenic mice will provide an important tool in the study of ER β -containing cells in the PVN.

In addition to the PVN, ER β -EGFP cells were found in the periaqueductal gray, parabrachial nucleus, NTS and the RVLM, all of which are involved in cardiovascular regulation (Aicher et al., 2000; Hayward, 2007). These findings are consistent with our recent observations that neurons in the RVLM bind estrogens and contain extranuclear ER β -ir (Wang et al., 2006b; Milner et al., 2008b). In the RVLM, estrogen via an ER β -mediated mechanism, regulates Ca⁺⁺ current inhibition of bulbospinal neurons which could contribute to a decrease in sympathetic tone (Wang et al., 2006b). Future physiological studies using *Esr2* BAC transgenic mice will help provide further insight into the function of the RVLM as well as other cardiovascular nuclei.

ER β -EGFP cells are found in brain areas associated with anxiety and stress

The present study found ER β -EGFP cells in many brain areas important for stress and anxiety behavior. Several studies have shown that ER β is involved in the modulation of monoaminergic system development with implications for anxiety behavior. Compared to wild-type mice, ER β knock out (ER β KO) mice have significantly lower levels of serotonin in the BNST, preoptic area, and hippocampus (Imwalle et al., 2005). In addition, male ER β KO animals show significantly less tryptophan hydroxylase mRNA expression in the dorsal raphe nucleus (Nomura et al., 2005). These brain areas are involved in stress and anxiety behavior and have been confirmed to contain ER β in the present study. Moreover, our study found that many ER β -EGFP neurons in the dorsal raphe contained serotonin. ER β KO animals show significantly less exploratory behavior in the open arm maze compared to wild-types, suggesting that ER β maybe be important for the development of normal anxiety behavior (Imwalle et al., 2005).

The current study found numerous ER β -EGFP cells in the hypothalamic PVN, supporting a growing body of literature showing an involvement of ER β in the PVN in stress reactivity. In particular, estrogen via an ER β mechanism regulates the expression of genes for CRF and urocortin, both of which are importantly involved in stress responses (Ogura et al., 2008; Haeger et al., 2006). Anatomical studies have demonstrated that ER β (protein or mRNA) colocalizes with both CRF and urocortin in the PVN (Haeger et al., 2006; Bingham et al., 2006; Laflamme et al., 1998). Additionally, during the stress response, estrogen is involved in restoring homeostatic balance in part by modulating nitric oxide production (Cherney et al., 2003; Gingerich and Krukoff, 2006). Recently, nitric oxide production in cultured rat hypothalamic neurons has been shown to be regulated via an ER β mechanism (Gingerich and Krukoff, 2008). Future studies crossing *Esr2* BAC transgenic mice with conditional ER β knock out animals are planned to further elucidate the role of ER β in select brain regions, such as the raphe or PVN, in anxiety behaviors.

ER β EGFP cells are in brain regions associated with learning and memory

Our finding that ER β -EGFP cells are in the cortex, hippocampal formation and olfactory bulb agrees with previous reports of ER β in these areas (Guo et al., 2001; Liu et al., 2008). ER β expression in these areas has been implicated in learning and memory. ER β agonists increase fast glutamate transmission and enhanced facilitation of long-term potentiation in hippocampal slices (Kramar et al., 2009), an effect that was not seen in ER β KO mice (Liu et al., 2008), and alter the ratio of AMPA receptor subunit expression in the hippocampus (Waters et al., 2009). ER β KO mice perform worse in hippocampal dependent memory tasks than wild types (Rissman et al., 2002; Walf et al., 2008a; Liu et al., 2008). In addition, ER β agonists improve memory and learning in rats and mice (Bodo and Rissman, 2006; Walf et al., 2008a). Our recent electron microscopic studies in the hippocampus have found that the synaptic distribution of ER β -ir is affected by estrogen levels (Waters et al., 2010). However, determining whether changes in ER β -ir occur in dendrites that contain other estrogen receptors or estrogen signaling molecules has been difficult using current methodologies. Thus, the use of Esr2 BAC transgenic mice in dual labeling electron microscopic immunocytochemical studies will fill an important gap.

The finding that ER β -EGFP cells are found in the subgranular zone of the dentate gyrus, is consistent with the localization of ER β mRNA and immunoreactivity in newly generated cells (i.e., cells undergoing adult neurogenesis) (Herrick et al., 2006; Isgor and Watson, 2005). ER β is an important regulator of cell proliferation in the adult dentate gyrus (Mazzucco et al., 2006). Cells undergoing adult neurogenesis have been linked to enhancements in performance on associative learning tasks (Shors, 2004; Snyder et al., 2005). Loss of a functional ER β also disrupts development and results in an under-population of neurons in the neocortex (Wang et al., 2003). In both the dentate gyrus and cerebral cortex, identifying ER β -containing dendrites has been difficult using immunocytochemical methods (Mitra et al., 2003; Shughrue and Merchenthaler, 2001). Thus, the detection of ER β -EGFP could advance the anatomical as well as physiological and molecular study of ER β -containing neurons in these regions.

ER β and other functions

The Esr2 BAC transgenic mouse line would be useful for studying ER β -containing cells in a number of brain regions implicated in a variety of other behaviors. For instance, ER β -EGFP labeling was found in prefrontal cortex (PFC), VTA, nucleus accumbens and subiculum, areas implicated in reward seeking and drug addiction (Hyman et al., 2006). While the VTA projects to the PFC, the nucleus accumbens and subiculum inputs to the VTA, in particular, subserves processes related to the learning of drug abuse (Vorel et al., 2001). Administration of the selective ER β agonist diarylpropionitrile (DPN) to OVXed female rats significantly increases cocaine-induced reinstatement response after a 14-day period of cocaine self-administration extinction (Larson and Carroll, 2007). This finding was discordant with the absence of effect seen following treatment with a selective ER α agonist, suggesting that ER β is involved in the mechanisms of addiction in women (Larson and Carroll, 2007). However, no evidence thus far suggests a role for ER β in addiction in males.

The present study also found ER β -EGFP cells in areas that modulate pain responses (e.g., the locus coeruleus and periaqueductal gray), and pain perception (e.g., the limbic system and hypothalamus). The observation that ER β KO mice have dampened pain responses (Spooner et al., 2007) suggests a functional role for ER β in these brain regions.

ER β -EGFP was expressed in areas that mediate sexually dimorphic outcomes. Hypothalamic ER β , particularly in the medial preoptic area, is implicated in female sexual behavior (Kudwa et al., 2006) but not in male sexual behavior (Nomura et al., 2006). Male

ER β knock-outs have increased aggression whereas female ER β KOs have decreased aggression (Ogawa et al., 2004). ER β -EGFP cells were observed in the medial amygdala; this region is crucial for the expression of sex-specific social behaviors and differs morphologically between female and male rats (Cooke et al., 2007;Cooke and Woolley, 2005). For example, female ER β KOs have impaired social discrimination when exposed to familiar and unknown individuals (Choleris et al., 2006). ER β -EGFP was also found in areas associated with feeding behavior, including the PVN, NTS, and parabrachial nucleus, supporting findings where ER β agonists induced socially acquired food preference learning while ER α agonists did not (Clipperton et al., 2008). All together, the evidence suggests that ER β has direct actions as well as the ability to counter ER α actions. ER β and ER α produce effects that both converge and diverge to generate a wide variety of responses in different brain areas. A difference in the receptor responses to estrogen and the limited availability of ER β selective ligands has hindered a deeper understanding of the hormonal mechanisms underlying physiology and behavior in animal models and humans. The availability of Esr2 BAC transgenic reporter mice presents a further opportunity to understand the involvement of ER β in these processes.

4. EXPERIMENTAL PROCEDURE

4.1 Animals

All methods were conducted in accordance with the 1996 National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Rockefeller University and Weill Cornell Medical College Institutional Animal Care and Use Committees. Adult female and male Esr2 BAC transgenic mice (N = 22; see below for description) were housed in a reversed light cycle (12:12 light:dark cycle, lights off 6am) temperature-controlled room with free access to food and water at the Rockefeller University. Female wild type C57BL/6 mice (N = 3; Jackson Labs, Bar Harbor, MI) and female Sprague Dawley rats (N = 4; Charles River Laboratories, Wilmington, MA) were housed on a normal light cycle, in a temperature controlled room with free access to food and water at Weill Cornell Medical College. Both mice and rats were between 2 and 3 months old at the time of sacrifice. The estrous stage of female mice and rats was monitored for two weeks prior to sacrifice using vaginal smear cytology (Turner and Bagnara, 1971). All Esr2 BAC transgenic mice were in diestrus (e.g., the low estrogen stage) on the day they were sacrificed. Wildtype mice and rats were used in our previous studies (Mitterling et al., 2010;Torres-Reveron et al., 2009;Torres-Reveron et al., 2008).

Hemizygous BAC-based Esr2-EGFP (ID169) mice were obtained from the GENSAT Project (www.gensat.org) at the Rockefeller University (Gong et al., 2003). Briefly, generation of the mice involved the insertion of an EGFP cassette into a BAC (RP23-342B14) via homologous recombination. The EGFP sequence was placed at the start site for Esr2 via the use a homology arm that is directly upstream of the ATG start site of the coding sequence of Esr2. The result is that the coding sequence of EGFP takes the place of the coding sequence of Esr2 on the BAC. The EGFP possess a poly A tail preventing the ER β gene on the BAC from being transcribed. Thus the cells expressing EGFP do not have more or less ER β expression than normal. The number of BACs that are inserted into the genome is usually in the range of 4-8 and insertion is limited to a single, random site. The native copy of the Esr2 gene on chromosome 12 of the mouse is untouched and functions as normal. Hemizygote BAC-based Esr2 transgenic mice were originally on a FVB/N background and have been back-crossed with wild-type C57BL/6 mice for three generations.

4.2 Immunocytochemical procedures

4.2.1 Section Preparation—Animals were deeply anesthetized with sodium pentobarbital (150 mg/kg) and their brains fixed by aortic arch perfusion sequentially with 5-7 ml saline (0.9%) containing 1000 units of heparin followed by 40 ml of 3.75% acrolein and 2% paraformaldehyde in PB (Milner et al., 2005). Following the perfusion, the brains were removed and cut into 5 mm coronal blocks using a brain mold (Activational Systems, Inc., Warren, MI), and post-fixed for 30 minutes in 2% acrolein and 2% paraformaldehyde in PB. The brains were sectioned on a Leica VT1000X Vibratome (40 μ m thick) and stored at -30°C in cryoprotectant (30% sucrose, 30% ethylene glycol in PB) until immunocytochemical processing. Prior to immunocytochemistry, sections were treated with 1% sodium borohydride in PB for 30 minutes to neutralize free aldehydes and rinsed thoroughly in PB. Sections then were rinsed in 0.1 M Tris-buffered saline (TS; pH 7.6) and incubated in 0.5% bovine serum albumin (BSA) in TS for 30 minutes.

4.2.2 Antisera—All antisera have been well characterized. Two antisera to ER β were employed: 1) A rabbit polyclonal antibody to ER β (485; Merck Research Laboratories, Rahway, NJ) was generated against a conserved sequence (rat aa 64-82) of the mouse, human, and rat ER β that is located within the A/B domain of ER β (exons 2-3) and is not present in ER α (Mitra et al., 2003). Previous studies have demonstrated the specificity of this antibody (Mitra et al., 2003; Milner et al., 2005). Briefly, immunoblots of total extract of SF9 cells transfected with cloned rat and mouse or human ER β identified a band migrating at $\sim 60\text{kDa}$, whereas no staining was detected in extracts of cells transfected with either rat or human ER α . Western blots showed, a band migrating at $\sim 55\text{kDa}$ in extracts of human ovary and human testes; the latter tissue also showed a band migrating at $\sim 70\text{kDa}$. Additionally, Western blots of whole brain extracts from rat and mouse showed a single band of $\sim 70\text{kDa}$. No bands were seen in brain extracts or extracts of SF9 cells expressing the cloned mouse, rat, or human ER β when 80424 was preincubated with the immunogenic peptide. COS-7 cells transfected with human ER β showed strong nuclear labeling with the 80424 antibody. This labeling was eliminated with preadsorption of the immunogenic peptide. Moreover, no staining was detected in COS-7 cells transfected with ER α or an empty vector. No immunoreactivity was detected in the hippocampal formation from mice and rat brains fixed with acrolein/paraformaldehyde (i.e., the same fix used in the present studies) when tissue was incubated without the primary antibody or with antisera preadsorbed with cognate peptide (Mitterling et al., 2010; Milner et al., 2005; Mitra et al., 2003). 2) An ER β antibody raised in rabbits (Z8P; Zymed Laboratories, San Francisco, CA) against a peptide sequence in the C-terminus (aa 468-485) of the mouse ER β protein (Shughrue and Merchenthaler, 2001). This antibody has been shown to be specific for ER β ($\sim 60\text{ kD}$) by Western blot analysis, double label with mRNA using *in situ* hybridization, preadsorption control and absence of labeling in fixed brain sections prepared from ER β knock-out mice (Shughrue and Merchenthaler, 2001; Creutz and Kritzer, 2002). In hippocampal rat brain tissue fixed with acrolein/paraformaldehyde, immunoreactivity with this antibody yields a similar subcellular localization as that seen with the Merck ER β antibody (Milner et al., 2005).

The chicken GFP antibody was generated against recombinant GFP (GFP-1020; Aves Lab Inc., San Diego, CA) and recognizes the gene product of EGFP expressing transgenic mice (Encinas et al., 2006). The specificity of this antibody has been demonstrated by Western blot (see data sheet for EGFP-1020 at www.aveslab.com) and has been used in our previous study (Bullock et al., 2008).

A polyclonal antibody to serotonin (5-HT) conjugated to bovine serum albumin (BSA) was generated in rabbits (Incstar Inc., Stillwater, MN). Previous studies have shown specificity

with preadsorption of the optimal dilution of the antibody with the cognate peptide (Milner and Veznedaroglu, 1993).

A polyclonal antibody to arginine-vasopressin (AVP) was generated in guinea pig (lot #061305; Peninsula Laboratories Inc., San Carlos, CA) and has been used in recent studies using identical labeling methods (Coleman et al., 2009). By radioimmunoassay, this antiserum shows complete recognition of vasopressin, a slight cross-reaction (<1%) with other vasopressin derivatives and no recognition of oxytocin (Peninsula Laboratories).

4.2.3 Immunoperoxidase methods—Coronal sections (approximately 240 μ m apart) through the rostrocaudal extent of the *Esr2* BAC transgenic mouse brain were processed for the immunocytochemical localization of the GFP antiserum using the avidin-biotin complex (ABC) method (Hsu et al., 1981). Tissue sections were incubated for 24 hrs at room temperature and for 24 hours at 4°C in the GFP antiserum (1:15,000 dilution) in 0.1% BSA in Tris-saline (TS; pH 7.6). All sections were incubated in a 1:400 dilution of biotinylated goat anti-chicken immunoglobulin (IgG) (Jackson ImmunoResearch Inc., West Grove, Pennsylvania) BSA/TS for 30 minutes. Following incubation in the secondary antibody, the sections were rinsed in TS and incubated in peroxidase-avidin complex (at twice the recommended dilution; Vector Laboratories; Burlingame, CA) for 30 minutes followed by development in 3,3'-diaminobenzidine (Aldrich Chemical Co., Milwaukee, Wisconsin) and H₂O₂ in TS for 6 minutes. Sections were mounted on slides previously coated with 1% gelatin and dried in a desiccator. The following day, the slides were dehydrated through a graded series of ethanols and coverslipped from xylene with DPX (Aldrich Chemical Co.).

Sections labeled for ER β -EGFP using immunoperoxidase were analyzed on a Nikon 80i light microscope equipped with bright-field and differential interference optics and a Micropublisher digital camera (Q Imaging, Barnaby, British Columbia). The distribution of ER β -EGFP cells was plotted onto standardized brain maps (Hof et al., 2000) using Adobe Illustrator CS3 (Adobe systems). Each map represents the relative distribution of ER β -EGFP cells using a composite of 8 brains (4 female and 4 male). EGFP containing cells were qualitatively divided into 3 groups: 1) dark cells (solid circles); 2) light cells (open circles); and 3) many dark cells (starbursts) (see Fig. 2). An additional set of sections was prepared for quantitative analysis. For this, sections from the rostrocaudal extent of 8 *Esr2* EGFP transgenic mice (4 diestrus females and 4 males) were run in parallel using the same batch of primary and secondary antibodies. ER β -EGFP cells from select brain regions then were counted from a 0.04 mm² area using a grid reticule on a Nikon Labophot. Differences between groups were determined using a student's t-test.

For comparison, some sections from the brains of wild-type mice or rats were processed for immunolabeling of ER β . For this, sections were incubated either in the Merck or Zymed ER β antibodies, both diluted 1:1000 for one day at room temperature (~21°C) and four days at 4°C. Sections then were processed for peroxidase immunocytochemistry as described above.

4.2.4 Dual labeling immunofluorescence methods—Sections through select brain regions were dually labeled for EGFP and either ER β , AVP or 5-HT, using immunofluorescence as previously described (Milner et al., 2008a). Procedures were the same as described above except that the ER β (Zymed antibody, 1:1000), AVP (1:1200) or 5HT antiserum (1:4000) was added to the primary antibody diluent one day prior to processing and the EGFP antibody was more concentrated (1:5000). Sections then were incubated sequentially for 1 hour each in Alexa 448 (green) species goat anti-chicken IgG for demonstration of EGFP (1:400; Invitrogen-Molecular Probes, Carlsbad, CA) and Cy5 (red) goat anti-guinea pig IgG for the demonstration of vasopressin and donkey anti-rabbit

IgG for demonstration of ER β and 5HT (1:400; all from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). For controls, sections were incubated in the absence of one or both of the primary antibodies and processed through the secondary antibodies. Sections were mounted on gelatin-coated slides, air-dried and coverslipped with Krystalon mounting media (Harleco EMD). Immunofluorescence images were acquired using a Nikon E75 fluorescence microscope equipped with *CY2*, *CY3*, *CY5*, *UV* and *YFP* filters.

4.2.5 Preparation of specimens for physiological or molecular studies—Fresh tissue slices and isolated cells were prepared from the PVN of female and male adult Esr2 BAC transgenic mice (N=6) using previously described methods (Li et al., 1998; Wang et al., 2006a; Wang et al., 2004). Four days prior to sacrifice, three of these mice were anesthetized with isoflurane (1.5%) in O₂ and microbeads conjugated to Texas red were injected bilaterally (1 μ l/side) into the interomediolateral region of the thoracic (T₂-T₄) spinal cord (Wang et al., 2006b; Wang et al., 2008). Mice were anesthetized with CO₂, decapitated, and their brains rapidly removed and immersed in artificial cerebrospinal fluid (aCSF). Coronal 200 μ m thick slices through the hypothalamus were cut using a vibratome and the PVN identified under bright-field microscopy using the lateral ventricle, fornix and optic chiasm as landmarks (Hof et al., 2000). Unenhanced EGFP-containing neurons as viewed under a Nikon epi-fluorescent microscope. For preparation of isolated cells, the PVN region was removed using a micro punch, and subjected to mild enzymatic digestion with 0.02% pronase for 1 hr and 0.02% thermolysin for 1 hr (Wang et al., 2006b; Wang et al., 2006a). Isolated PVN neurons were attached to a glass slide, and perfused with lactic acid in aCSF.

4.2.6 Dual labeling immunoelectron microscopic methods—For electron microscopic localization of ER β -ir in Esr2 BAC transgenic mice, sections through select brain regions were dual-labeled for ER β -immunoperoxidase and EGFP-immunogold using methods described previously (Bulloch et al., 2008). Briefly, sections were incubated in the Merck anti-ER β antiserum (1:1,000) in 0.1% BSA in TS for 1 day at room temperature followed by an additional 4-5 days at 4°C. One day prior to further processing, EGFP antiserum (1:5000) was added to the primary antibody diluent. To visualize ER β -immunoreactivity, sections were incubated in biotinylated goat anti-rabbit IgG (Vector) and processed for the ABC peroxidase technique described above. To visualize EGFP-labeling, sections were rinsed in TS and incubated in rabbit anti-chicken IgG conjugated to 1 nm gold particles (1:50, Electron Microscopy Sciences, Fort Washington, PA) in 0.08% BSA and 0.01% gelatin in 0.01M phosphate-buffered saline (PBS; pH 7.4) at room temperature for 2 hours. Sections then were rinsed in PBS, post-fixed in 2% glutaraldehyde in PBS for 10 minutes, and rinsed in PBS followed by 0.2 M sodium citrate buffer (pH 7.4). The conjugated gold particles were enhanced by treatment with silver solution (EMS) for 5 - 7 minutes.

Sections prepared for electron microscopy were post-fixed for 1 hour in 2% osmium tetroxide, dehydrated through alcohols and propylene oxide, and embedded between two sheets of plastic in EMbed 812 (EMS). Ultrathin sections (70 nm thick) were collected from the region of interest and cut on an ultratome (Ultracut, Leica). Sections were counter-stained with uranyl acetate and Reynold's lead citrate, and final preparations were analyzed on a FEI Tecnai Biotwin transmission electron microscope. Profiles were identified and categorized using standardized morphological characteristics (Peters et al., 1991).

4.2.7 Image preparation—Final photomicrographs were generated from digital images adjusted for levels, brightness and contrast in Adobe Photoshop CS3 on a Macbook computer. Images were assembled into the final figures using Powerpoint 2004.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ABC	avidin biotin complex
AC	anterior cingulate
AQ	aqueduct
AVP	arginine vasopressin
BLA	basolateral amygdala
BSA	bovine serum albumin
cc	corpus callosum
CeA	central nucleus of the amygdala
CRF	corticotropin releasing factor
dg	dentate gyrus
DR	dorsal raphe nucleus
DRN	dorsal reticular nucleus
DMX	dorsal motor nucleus of vagus
EGFP	enhanced green fluorescent protein
EP	endopiriform nucleus
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
5-HT	5 -hydroxytryptamine
gcl	granule cell layer
hil	hilus of the dentate gyrus
LS	lateral septum
ml	molecular layer
mlf	medial lateral fasciculus
MS	medial septum
NA	nucleus accumbens
NR1	NMDA receptor subunit 1
NTS	nucleus of the solitary tract

PAG	periaqueductal gray
pcl	pyramidal cell layer
PB	phosphate buffer
PBL	parabrachial nucleus
PFC	prefrontal cortex
PVN	paraventricular nucleus of the hypothalamus
SIG	silver intensified gold
SLM	stratum lacunosum-moleculare
SO	stratum oriens
SR	stratum radiatum
SUB	subiculum
Rt	reticulothalamic nucleus
RVLM	rostral ventrolateral medulla
TH	tyrosine hydroxylase
ts	tractus solitarius
TS	tris saline
VTa	ventral tegmental nucleus

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Fig. 1. Morphology EGFP cells in the brains Esr2 BAC transgenic mice

A-D. As shown in these examples from fixed tissue sections, EGFP peroxidase reaction product is visible in the nuclei (arrowheads), cytoplasm and processes (arrows) of cells having the morphology of neurons. Photographs from ventral subiculum (A), layer 5 of the somatosensory cortex (B), the piriform cortex (C), and the endopiriform nucleus (D). **E-I.** Example of a 200 µm thick fresh-tissue coronal slice and dissociated cells from the PVN of an Esr2 BAC transgenic reporter mouse prepared for physiological studies viewed under bright field and fluorescence microscopy. **E.** Under bright field, landmarks [e.g., the fornix (fx) and third ventricle (3v)] identifying the PVN can be distinguished. **F.** With fluorescence microscopy, a dense cluster of cells with intense unenhanced EGFP (identified by green autofluorescence) can be distinguished in the PVN. **G.** In a 200 µm thick fresh-tissue coronal slice from an animal injected in the spinal cord with Texas red, EGFP neurons (green) colocalize (yellow) with retrogradely transported Texas Red (red). **H.** When visualized under bright-field, individual healthy cells (arrows) can be seen in the petri dish. **I.** When viewed with fluorescence microscopy, native EGFP is visible in many of these cells. Bars A-D and G and H, 25 µm, E and F, 0.5 mm

Fig. 2. Distribution of EGFP cells in coronal sections of the Esr2 BAC transgenic mouse brain
Drawings are arranged from rostral (A) to caudal (M) and are separated by a distance of 500 – 800 μ m. The drawings are modified from Hof et al. (2000) and represent a composite qualitative distribution of the brains from 4 male and 4 female Esr2 BAC transgenic mice. In select regions, the number of cells was quantified (see table 1). Bar, 1 mm. Abbreviations for figure 1 are appended.

Fig. 3. Distribution of ER β -EGFP labeling in select forebrain regions

A. The lateral septum (LS) contains numerous, densely packed ER β -EGFP cells. Scattered ER β -EGFP cells are found in the medial septum (MS) and the core of the nucleus accumbens (ACB). **B.** Several ER β -EGFP cells are found in the anterior portion of the basolateral amygdala (BLA) and dorsal endopiriform nucleus (EPd) whereas almost no ER β -EGFP cells are seen in the central nucleus of the amygdala (CeA). **C.** The lateral habenula (LH) harbors a dense accumulation of ER β -EGFP cells. **D.** A dense band of ER β -EGFP cells is found in the reticulothalamic nucleus (Rt). BLV, ventral portion of the basolateral amygdala; CP, caudate-putamen; MH, medial habenula; st, stria terminalis. Bar, 100 μ m.

Fig. 4. Distribution of ER β -EGFP cells in the paraventricular nucleus of the hypothalamus
A. Numerous ER β -EGFP containing cells are found in the PVN. Higher magnification (inset) shows that dendritic processes (arrow) of PVN neurons can often be identified. **B.** Dual labeling reveals that the majority of ER β -EGFP cells are located medial to and interspersed with magnocellular neurons identified with AVP-ir. **C & D.** The distribution of cells containing ER β -immunoreactivity (-ir) in both mouse (**C**) and rat (**D**) is similar to that of EGFP cells in the *Esr2* BAC transgenic mice. ER β -ir is found in both the nuclei and cytoplasm of PVN cells. Compared to rat, ER β -EGFP and ER β -immunoreactive cells are diffusely distributed throughout the mouse PVN. Fornix, fx. Bar A-D, 0.5 mm; E, F & G, 50 μ m; Inset, 50 μ m

Fig. 5. Distribution of ER β -EGFP in the cerebral cortex and hippocampus

A. At low magnification, numerous ER β -EGFP cells are found in the cerebral cortex in layer 5 and overlying the corpus callosum (cc) as well as the anterior cingulate cortex (AC) and subiculum (SUB). **B.** At higher magnification, many cells in layer 5 of the cortex have the morphology of pyramidal cells and contain EGFP reaction product in their dendritic processes (arrows). ER β -EGFP cells near the corpus callosum are often clustered (arrowheads). **C.** In the CA1 region of the dorsal hippocampus, intensely labeled ER β -EGFP cells (arrowheads) are scattered near the pyramidal cell layer (pcl). Diffuse ER β -EGFP labeling is found throughout the other cell layers, but is most intense in stratum lacunosum-moleculare (SLM). **D.** In the caudal aspects of the hippocampus, numerous ER β -EGFP cells are seen in the hilus (hil) of the dentate gyrus (dg). **E.** At higher magnification, the ER β -EGFP cells have numerous labeled processes (example, arrows). gcl, granule cell layer; ml, molecular layer; SO, stratum oriens; SR, stratum radiatum. Bar, 100 μ m

Fig. 6. Distribution of ER β -EGFP cells in select pontine nuclei

A. In the dorsal raphe (DR), numerous ER β -EGFP cells are detected. **B.** Numerous ER β -EGFP cells are detected in the lateral parabrachial nucleus (PBI). **C.** In sections of the raphe magnus from *Esr2* BAC mice dually labeled with 5-HT (red), numerous cells contain both markers (yellow). For comparison, cells showing only ER β -EGFP (green; arrows) are shown. AQ, aqueduct; mlf, medial lateral fasciculus; PAG, periaqueductal gray. Bar, 100 μ m

Fig. 7. Distribution of ER β -EGFP cells in select medullary nuclei

A. Numerous ER β -EGFP cells are found in the medial nucleus of the solitary tract (mNTS). A few large lightly labeled ER β -EGFP cells are found in the dorsal motor nucleus of the vagus (DMX). **B.** Scattered ER β -EGFP cells are found in the rostral ventrolateral medulla (RVLM). **C.** Numerous scattered ER β -EGFP cells are observed in the spinal nucleus of the trigeminal nerve (SPVC). AP, area postrema; cc, central canal; mDRN, medial dorsal reticular nucleus; scp, superior cerebellar peduncle; ts, tractus solitarius. Bar, 100 μ m

Fig. 8. Light microscopic localization of ER β -EGFP and ER β -immunoreactivity in select brain regions

Sections through the PVN (**A**), medial amygdala (**B**), dorsal raphe (**C**), the caudal medial NTS (**D**) and CA3 region of the hippocampus (**E**) were dually labeled for GFP (green; photograph 1) and ER β -ir (red; Zymed Z8P antibody; photograph 2). Merged images (photograph 3) show that most EGFP-containing cells contain ER β -immunoreactive nuclei in all five brain regions. In the caudal medial NTS (**D**) and the CA3 region of the hippocampus (**E**) and, cytoplasmic ER β -ir was detected in some EGFP-containing cells (arrows). Bar, 100 μ m.

Fig. 9. Electron microscopic localization of ER β -EGFP in perikarya in the PVN

A. Peroxidase labeling for EGFP from an Esr2 BAC transgenic mouse is distributed throughout the nucleus (N) and cytoplasm of perikarya. **B.** In PVN sections from an Esr2 BAC transgenic mouse dually labeled for EGFP (immunogold silver) and ER β immunoperoxidase, ER β -EGFP SIG particles (black dots) are dispersed throughout the nucleus and cytoplasm whereas ER β -ir is found in small patches in the nucleus (bottom arrow) and on the plasma membrane (top arrow). er, endoplasmic reticula; m, mitochondria; nu, nucleolus; Bars, 500 nm.

Fig. 10. Electron microscopic localization of ERβ-EGFP in dendrites in the PVN

A & B. Peroxidase labeling for ERβ-EGFP is found throughout the cytoplasm of large (**A**) and small (**B**) dendritic profiles. Both types of EGFP-labeled dendritic profiles, are contacted by unlabeled terminal (uT). **C.** In the PVN from an Esr2 BAC transgenic mouse dually labeled for EGFP (immunogold-silver) and ERβ (immunoperoxidase), ERβ-EGFP SIG particles (black dots) are dispersed throughout the cytoplasm whereas ERβ-ir is found in small patches (arrowheads) near mitochondria (m). Bars, 500 nm.

Fig. 11. Electron microscopic localization of ERβ-EGFP in the medial amygdala

Sections from the medial amygdala of *Esr2* BAC transgenic mice dually labeled for GFP (immunogold-silver) and ERβ immunoreactivity (immunoperoxidase). **A.** EGFP SIG particles (black) are found in both the nucleus (N) and the cytoplasm whereas ERβ-immunoperoxidase is found in patches (arrowheads) within the nucleus of one cell (bottom) and in the cytoplasm of another (top cell). **B.** In this dendritic shaft profile, EGFP SIG particles (black) are found throughout the cytoplasm whereas ERβ-immunoperoxidase is more discretely localized (arrowheads) near mitochondria (m) and endoplasmic reticula (er). Bars, 500 nm.

Fig. 12. Electron microscopic localization of ER β -EGFP in stratum radiatum of the CA1 region of the hippocampus

A & B. In the CA1 region of an *Esr2* BAC transgenic mouse EGFP peroxidase reaction product found throughout a dendritic (D) profile contacted by unlabeled terminals (uT) (A) and in an axon (ax) profile (B). **C & D.** Photomicrographs from the CA1 region of an *Esr2*-EGFP BAC transgenic mouse dually labeled for EGFP and ER β . A high magnification (C), ER β -ir (identified using immunoperoxidase) is found in a dendritic spine (arrowhead) that is contacted by an unlabeled terminal (uT). At low magnification (D), the spine with ER β -ir shown in C (boxed region) is found in a dendritic profile that contains EGFP SIG particles scattered throughout the cytoplasm. uD, unlabeled dendrite Bars, 500 nm.

Fig. 13. Electron microscopic localization of ER β -EGFP in the raphe magnus

A. Both nuclear and extranuclear ER β -ir (identified using immunoperoxidase; arrows) are found in an EGFP labeled perikaryon (identified by immunogold-silver; chevrons) from an *Esr2* BAC transgenic mouse. ER β -ir appears as discrete patches throughout the nucleus (N) and cytoplasm where it is sometimes near (arrows) mitochondria (m). EGFP SIG particles are found throughout the nucleus and cytoplasm. nu, nucleolus Bars, 500 nm.

Table 1

Comparison of number of ERb-EGFP cells in select brain regions

Brain region with map reference	Female (N = 4; * p< 0.05)	Male (N = 4)
Lateral septum (Fig. 2B)	11.1 ± 0.4*	8.3 ± 1.0*
Hypothalamic PVN (Fig 2D)	17.0 ± 1.3	15.0 ± 2.6
Medial amygdala (between Fig. 2D & E)	10.3 ± 1.5	7.25 ± 1.1
Lateral amygdala (Fig. 2E)	7.8 ± 1.0*	4.0 ± 1.3*
Endopyriform cortex (Fig. 2E)	12.5 ± 1.2	5.8 ± 0.6
Somatosensory cortex, layer 5 (Fig. 2E)	9.5 ± 1.8*	9.5 ± 0.7*
Dorsal subiculum (Fig. 2F)	17 ± 2.7	14.8 ± 1.4
Raphe magnus (Fig. 2J)	10 ± 1.5	8.5 ± 0.9