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Research report

Cells containing immunoreactive estrogen receptor-α in the human basal forebrain

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Abstract

The distribution of estrogen receptor protein-α (ER-α)-containing cells in the human hypothalamus and adjacent regions was studied using a monoclonal antibody (H222) raised against ER-α derived from MCF-7 human breast cancer cells. Reaction product was found in restricted populations of neurons and astrocyte-like cells. Neurons immunoreactive for ER-α were diffusely distributed within the basal forebrain and preoptic area, infundibular region, central hypothalamus, basal ganglia and amygdala. Immunoreactive astrocyte-like cells were noted within specific brain regions, including the lamina terminalis and subependymal peri-third-ventricular region. These data are consistent with the location of estrogen receptors in the basal forebrain of other species and the known effects of estrogens on the cellular functions of both neurons and supporting elements within the human hypothalamus and basal forebrain.

Keywords: Estrogen receptor-α; Immunocytochemistry; Hypothalamus; Basal forebrain; Human

1. Introduction

Gonadal steroids have profound effects on the development, maintenance and induction of various behavioral patterns in vertebrates [4,8,44,58]. Studies with rats have shown that sexual differentiation of the nervous system only occurs after steroid exposure during a certain critical period in development, effecting changes that are both organizational and permanent [27]. Many studies over the last 30 years have shown that steroids act on neurons by binding to specific hormone receptor molecules contained in neurons [38]. Some of the areas that are implicated for the control of hormone-dependent behavior are known to contain hormone-binding neurons in several vertebrate species' brains [4,8,44,58]. Methods used for localizing gonadal steroid-binding neurons in the brain have included autoradiography with tritium-labeled steroids [45,61,66] and immunocytochemical localization of receptor-bound steroids [2,5,6,20,47].

It has been shown by in situ hybridization that neurons within the human hypothalamus do express estrogen receptor-α mRNA [48]. However, the distribution of neurons that express estrogen receptor protein-α (ER-α) in the human hypothalamus has yet to be mapped. Monoclonal antibodies to ER-α have demonstrated great means of detection of ER-α; immunocytochemistry utilizing such

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monoclonal antibodies has demonstrated great sensitivity and resolution, as well as highly specific labeling of the receptors [21–23,30].

The present study uses a rat monoclonal antibody directed against the ligand-binding site of human ER-α (H222) [23]. These monoclonal anti-ER-α antibodies that were derived from MCF-7 human breast cancer cells were shown to provide a useful system for the immunocytochemical demonstration of estrogen receptors in individual cancer cells [21,23]. The H222 antibody has been shown to be specific for the ER-α subtype in multiple mammalian species [20,33,47].

2. Materials and methods

2.1. Human tissues

The four adult human brains used in this study were obtained within 2–12 h post-mortem. All of the brains were subjected to neuropathologic examination and found to be histologically normal. All subjects for this current study had no history of neurologic or endocrine disease. There were two male and two female brains from subjects with an age range of 24–76 years. Both female brains were obtained from post-menopausal women.

2.2. Fixation and tissue preparation

A full coronal block taken rostral to the optic chiasm and extending caudally to immediately behind the mammillary bodies was subsequently trimmed to include the entire hypothalamus/basal forebrain and adjacent left basal ganglia and amygdala. The trimmed fresh brain tissue was then sliced coronally at the level of the median eminence. The two halves, which measured approximately 1 cm in thickness, were fixed by immersion in Zamboni's fixative or 4% paraformaldehyde (EM grade) at 4°C for 12 h. Two fixatives were used in order to determine whether there would be differences in immunolabel observed between the two protocols. To prevent ice artifact, brain tissues were then immersed in 30% sucrose in 0.1 M phosphate buffer at 4°C until they sank to the bottom of the container (~15 h). Tissues were then snap frozen with liquid nitrogen and stored at −70°C in sealed plastic bags until sectioning (~1 week). The frozen brain tissues were placed on a liquid CO_2 stage and serially sectioned at 40 μm with a sliding microtome. These unmounted, ''free-floating'' sections were then placed in 0.1 M phosphate buffer at room temperature prior to pretreatment.

2.3. Immunocytochemistry

Sections were then pretreated with 0.01 M sodium metaperiodate in Tris-buffered saline (TBS: pH 7.6) for 15 min and 1% sodium borohydride in TBS for 10 min to remove residual aldehydes [2,9]. Sections were then permeabilized with 1% Triton x-100 followed by 5% dimethyl sulfoxide (both in TBS). Dimethyl sulfoxide is a membrane disruptor which was used to enhance tissue permeabilization. The rather high concentration of Triton x-100 was used because of the somewhat thick tissue sections (40 μm) and because the expected localization of ER-α was expected to be nuclear, which would require antibody penetration of both the plasma and nuclear membranes. Sections were subsequently incubated for 30 min in TBS with 3% hydrogen peroxide and 1% bovine serum albumin to deplete endogenous peroxidase activity and to decrease non-specific staining, respectively. The sections were incubated for 72 h in primary antibody, H222, at a concentration of 10 μg/ml and 1:100 dilution (Abbott Laboratories, North Chicago, IL) in TBS containing 0.5% Triton x-100, 0.1% gelatin and 0.02% sodium azide at a pH of 7.6 at 4°C. Other sections from these same areas were stained for endogenous peroxidase alone and used as controls. Optimal immunostaining of cytoplasmic processes required a fairly high concentration of primary antibody (10 μg/ml, 1:100 dilution) and of the secondary antiserum (1:10–1:25 μg/ml), plus the use of multiple bridges [64]. The multiple bridge technique has been used extensively in previous studies of estrogen and progestin receptors in the central nervous systems of other species; identical results have been reported with both this technique and the avidin–biotin complex technique [6,9,34].

The sections were then incubated for 30 min at room temperature in secondary antiserum (goat anti-rat immunoglobulins: American Qualex, La Miranda, CA). The sections were subsequently incubated for 1/2-h in tertiary antibody (rat peroxidase–antiperoxidase complex: Sternberger-Meyer Immunocytochemical, Jarrettsville, MD) at a concentration of 1:100. In the multiple bridge technique, sections were returned to bridging secondary antiserum and then peroxidase–antiperoxidase complex three to five times. Finally, sections were incubated with diaminobenzidine (0.05% in TBS; Sigma, St. Louis, MO) in the presence of 0.05% hydrogen peroxide to produce a brown reaction product. Sections were then washed in 0.05 M Tris buffer, mounted on glass slides, allowed to air dry, and coverslipped with Permount.

Previous studies which have demonstrated the specificity of the monoclonal antibody H222 for estrogen receptors in guinea pigs have included preadsorption of the antibody with an estrogen receptor-rich cellular extract [7], omission of the primary antibody, and acute pretreatment of animals with estradiol to decrease estrogen receptor immunoreactivity (ERIR) [9]. Specificity of the antibody has been demonstrated by preadsorption with estrogen receptor-rich extracts in rats [10], opossums [16], musk shrews [14] and sheep [33]. In the present study, omission of either the primary or secondary antibodies was noted to completely eliminate all specific ERIR.

2.4. Mapping

Line drawings of nuclei and myelinated structures were made from the original sections visualized by light microscopy, depicting ERIR cells at different levels (coronal sections) throughout the human hypothalamus. In addition, the Alsop-Riley atlas [1] was used to determine the location of those structures not indicated in the light microscopic drawings but described in the text.

3. Results

3.1. Estrogen receptor-α immunoreactive cells and processes

The morphology in all of the four brains studied allowed clear delineation of immunopositive elements and reference structures at the light microscopic level. The presence of immunoreactive estrogen-receptor protein-α (ERIR) was indicated by dense brown granular reaction product. The criterion chosen for mapping of ERIR cells was the presence of reaction product in the cell nucleus and surrounding cytoplasm of the cell soma.

Examination of the cells in each area revealed that the soma and processes of many, but not all of these cells were immunostained (see Figs. 1–3 and 5–7). They tended to be medium-sized and multipolar. The largest amount of reaction product was observed in the cell nucleus. A paler area within the nucleus was consistently observed which corresponded to the nucleolus. Large amounts of reaction product were also observed in the cytoplasm of the cell soma, sometimes approaching that of the nucleus. Some of the neuronal processes (axons and dendrites) also exhibited reaction product, albeit less than in the nucleus and surrounding cytoplasm. These features are similar to those of

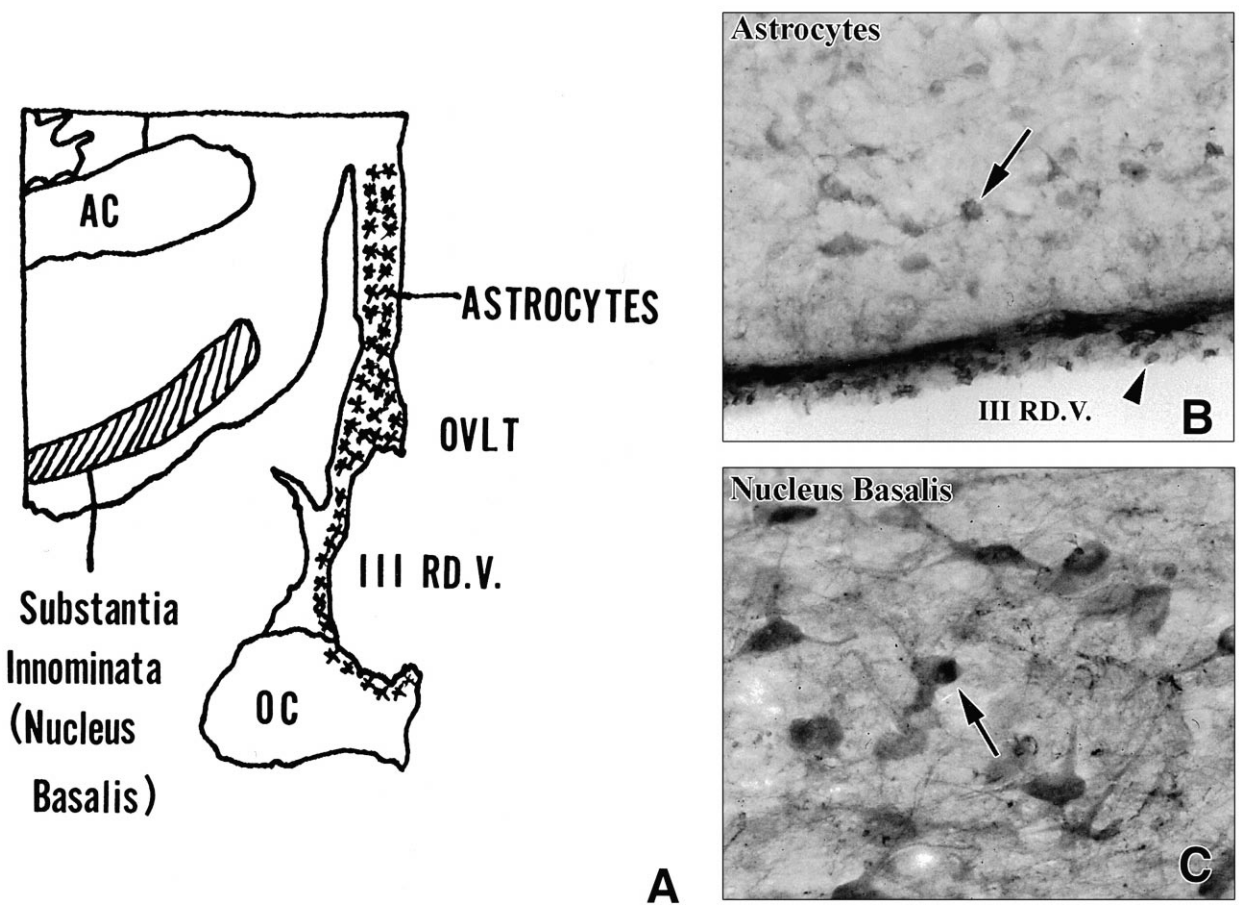

Fig. 1. Schematic diagrams illustrating the location of ERIR neurons and astrocytes throughout coronal sections of the hypothalamus and adjacent areas in the human brain with accompanying photomicrographs. Diagrams of coronal sections proceed sequentially from anterior to posterior. (A) Schematic diagram showing ERIR neurons present in the substantia innominata (cross-hatching). ERIR astrocytes (stars) are seen adjacent to the third ventricle (III RD.V.) and in the organum vasculosum of the lamina terminales (OVLT). AC = anterior commissure, OC = optic chiasm. (B) Photomicrograph showing immunoreactive periventricular astrocytes beneath the ependymal surface of the third ventricle (arrow). The staining is strongest in the nucleus and cytoplasm of the cell body, though the cellular processes also demonstrate some staining. The ependymal cells (tanycytes) lining the ventricle are staining intensely (arrowhead). ×200. (C) Photomicrograph showing nuclear staining within large neurons of the nucleus basalis of Meynert (arrow). Staining is strongest in the nucleus, followed by the cytoplasm of the cell body. Areas of paler staining within nuclei likely correspond to the nucleoli. Positive-staining axons can also be seen coursing through the neuropil. ×200.

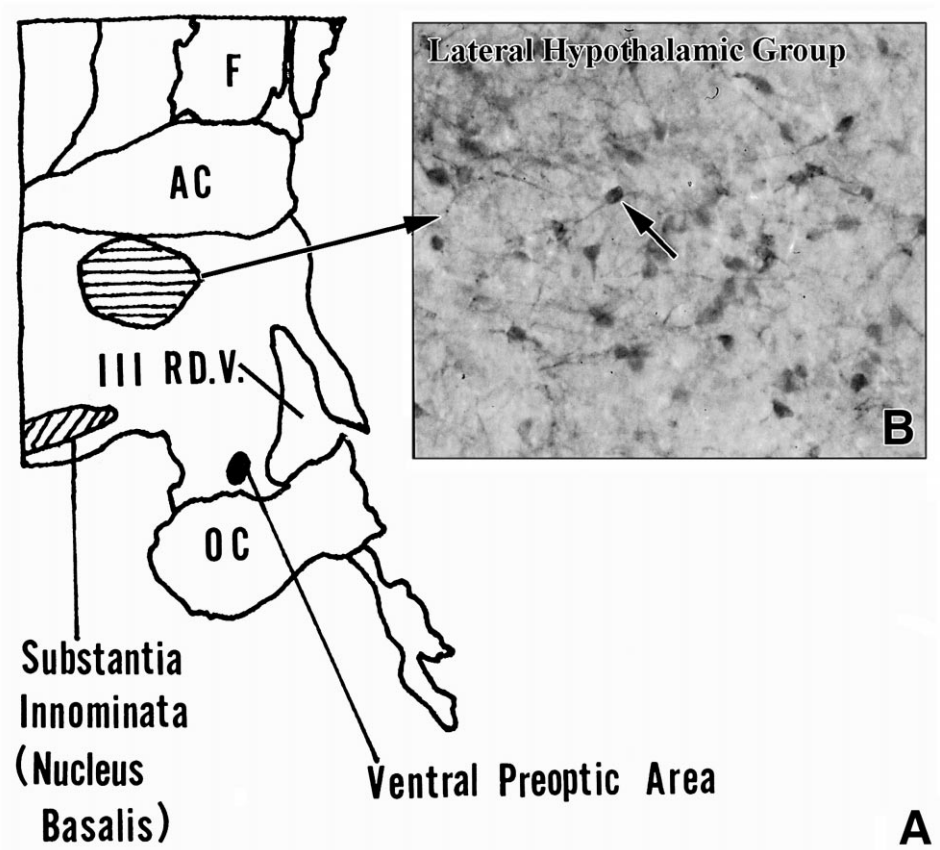

Fig. 2. (A) Schematic diagram showing ERIR neurons present in the lateral hypothalamic group (horizontal cross-hatching), substantia innominata (diagonal cross-hatching) and the infundibular group-ventral preoptic area (solid circle). F = fornix, AC = anterior commissure, OC = optic chiasm, III RD.V. = third ventricle. (B) Photomicrograph showing prominent nuclear and cytoplasmic staining within neurons of the lateral hypothalamic group (approximately the same area as the bed nucleus of the stria terminalis in the rat). The neurons are smaller than those of the nucleus basalis and about the same size as those of the infundibular and posterior hypothalamic groups (arrowhead). Neuronal processes are also clearly staining. Areas of paler staining within nuclei likely correspond to the nucleoli. ×200.

ERIR neurons described in the guinea pig [9], hamster [34] and ferret [57,59].

The other major general observation was the finding of ERIR within numerous periventricular, subependymal astrocyte-like cells and their stellate processes, and also within certain ependymal tanycytes lining the third ventricle (Fig. 1B). As with ERIR neurons, the reaction product within the astrocyte-like cells was largest in the nucleus and cytoplasm, with the processes containing less product. These results are consistent with the finding of ERIR glia in the guinea pig [32].

Examination of the regional distribution of immunoreactive cells and processes was performed on the large tissue sections sequentially obtained from all four adult human brains. No readily discernable differences in the distribution of reactive cells and fibers were observed in the four cases studied. There were also no detectable differences in reactivity between tissues fixed in either Zamboni's fixative or 4% paraformaldehyde.

In contrast to H222-treated sections, the sections stained for endogenous peroxidase alone showed no evidence of staining in neuronal cell bodies. Therefore, this specific staining could not be explained on the basis of reaction with endogenous peroxidase alone. Focal endogenous peroxidase reactivity was observed in occasional periventricular glial cells as previously described [41]. These cells were rare in control sections, indicating that the H222 glial immunoreactivity on antibody-treated sections cannot be explained on the basis of endogenous peroxidase activity alone.

3.2. Regional distribution of ER-α IR cells

The details of the distribution of ERIR cells are described with reference to five levels (Figs. 1–5). These include: Level 1, the organum vasculosum of the lamina terminalis (OVLT) and rostral preoptic area; Level 2, the caudal limit of the optic chiasm and the rostral periventricular region; Level 3, the infundibulum at the level of the crossing of the anterior commissure; Level 4, the anterior pillars of the fornix; and Level 5, the mammillary region. Sections for mapping were chosen on the basis of their

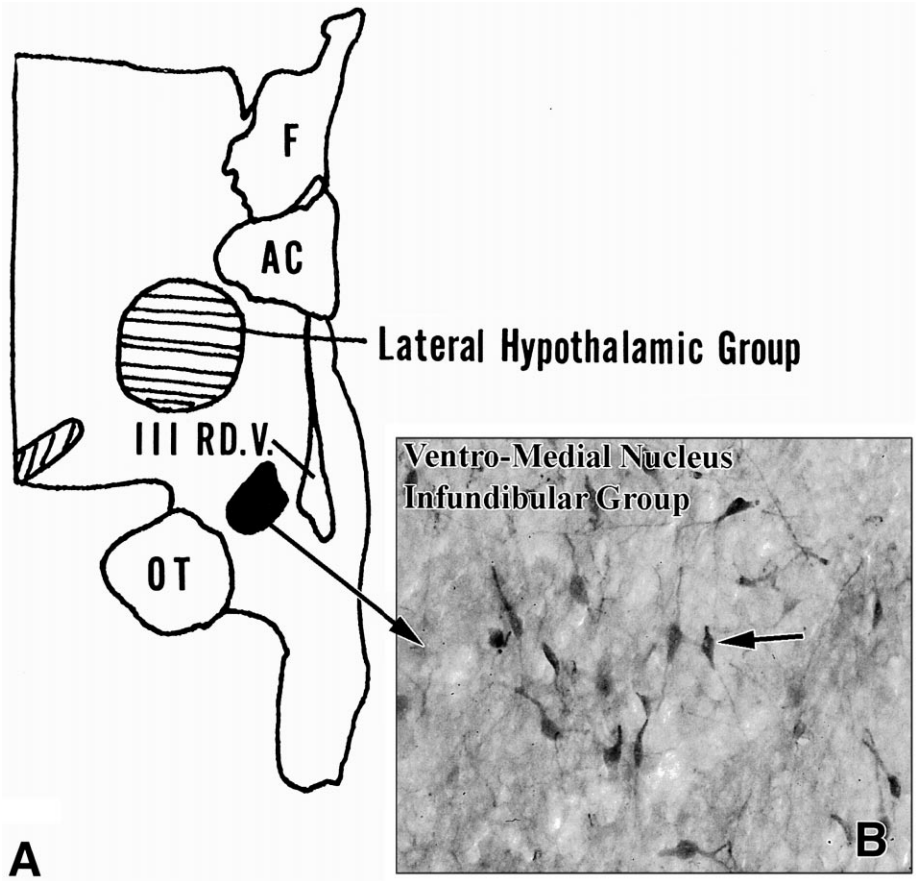

Fig. 3. (A) Schematic diagram showing ERIR neurons present in the lateral hypothalamic group (horizontal cross-hatching), the remnant of the substantia innominata (diagonal cross-hatching) and the infundibular group-ventromedial nucleus (solid circle). F = fornix, AC = anterior commissure, OT = optic tract, III RD.V. = third ventricle, I = infundibulum. (B) Photomicrograph showing neurons of the infundibular group. They are smaller than neurons of the nucleus basalis and approximately the same size as those of the lateral and posterior hypothalamic groups (arrowhead). Note the strong nuclear and cytoplasmic staining. The neuronal processes are also staining, though less intensely. Areas of paler staining within nuclei likely correspond to the nucleoli. ×200.

correspondence with plates T4-2152, T4-2066, T4-1954, T4-1874 and T4-1765 in the Alsop-Riley atlas [1]. The pituitary gland was not included in the present study, as the stalks were transected at the time of brain removal.

3.2.1. Level 1: OVLT and rostral preoptic area

A large concentration of ERIR cell bodies was found in the rostral preoptic area, particularly within the region lateral to the OVLT (Fig. 1). This anatomically corresponds to the nucleus basalis of Meynert in the substantia innominata. These neurons tended to be relatively large (compared to other groups to be shown) and multipolar. Another large concentration of ERIR cell bodies was seen in the OVLT along the third ventricle, extending down to the optic chiasm; these were ERIR cells which resembled astrocytes.

3.2.2. Level 2: caudal limit of the optic chiasm and rostral periventricular region

ERIR cell bodies continued to be demonstrated within the preoptic area (nucleus basalis); however, this area was beginning to get smaller as the sections became more posterior (Fig. 2). At this level, ERIR cell bodies of the lateral hypothalamic group began to appear just below the anterior commissure, and the infundibular group (ventral preoptic area) was first seen just above the optic chiasm and lateral to the third ventricle. Anatomically, the human lateral hypothalamic group is in approximately the same location as the bed nucleus of the stria terminalis in the rat. Both of these groups will become better established at the next level. ERIR periventricular astrocyte-like cells were no longer seen and were not seen in any of the more posterior sections.

3.2.3. Level 3: infundibulum at the level of the crossing of the anterior commissure

Both the lateral hypothalamic and infundibular groups of ERIR cell bodies were much larger than in the previous level (Fig. 3). The infundibular group now corresponds to the ventromedial nucleus in both this level and the next more posterior level. Compared with the nucleus basalis, the ERIR neurons of the infundibular group were relatively

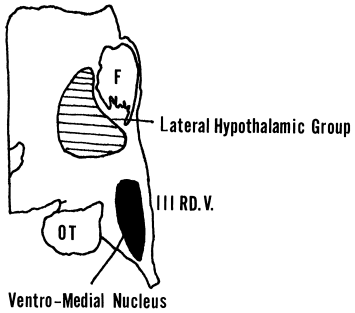

Fig. 4. Schematic diagram showing ERIR neurons present in the lateral hypothalamic group (horizontal cross-hatching) and the infundibular group (large solid area). No new groups are demonstrated at this level. F = fornix, OT = optic tract, III RD.V. = third ventricle.

small and multipolar; this group was seen just lateral to the third ventricle and rostromedial to the optic tract. The ERIR neurons of the lateral hypothalamic group tended to be similar in size to those of the infundibular group and also multipolar. At this level, this group was located just lateral and caudal to the anterior commissure. The size of the nucleus basalis was getting smaller as the sections got more posterior.

3.2.4. Level 4: anterior pillars of the fornix

Both the infundibular (ventromedial nucleus) and lateral hypothalamic groups were larger, while the nucleus basalis had disappeared (Fig. 4). The infundibular group was now just lateral to the third ventricle and medial to the optic tract. The lateral hypothalamic group was just lateral and caudal to the fornix.

3.2.5. Level 5: mammillary bodies

ERIR cell bodies were seen in the posterior hypothalamus, surrounding the mamillothalamic tract (tract of Vicq d'Azyr), and in the mamillary body itself (caudal to the posterior hypothalamic group) (Fig. 5). These neurons were multipolar; they were smaller than neurons of the nucleus basalis and about the same size as those of the lateral hypothalamic and infundibular groups. Their staining characteristics were similar to all of the other ERIR cell bodies identified. None of the previously identified groups of neurons could be seen at this level.

3.2.6. Other: amygdala and globus pallidus

ERIR cell bodies were seen in the amygdala (Fig. 6) and in the globus pallidus (Fig. 7). The reaction product in these neurons was similar to those observed in the hypothalamus. The neurons of the amygdala were very small compared with those in the hypothalamus, and their processes were not well visualized. A few of the globus pallidus nuclei contained large amounts of reaction product. They were multipolar and similar in size to the hypothalamic neurons but larger than those of the amyg-

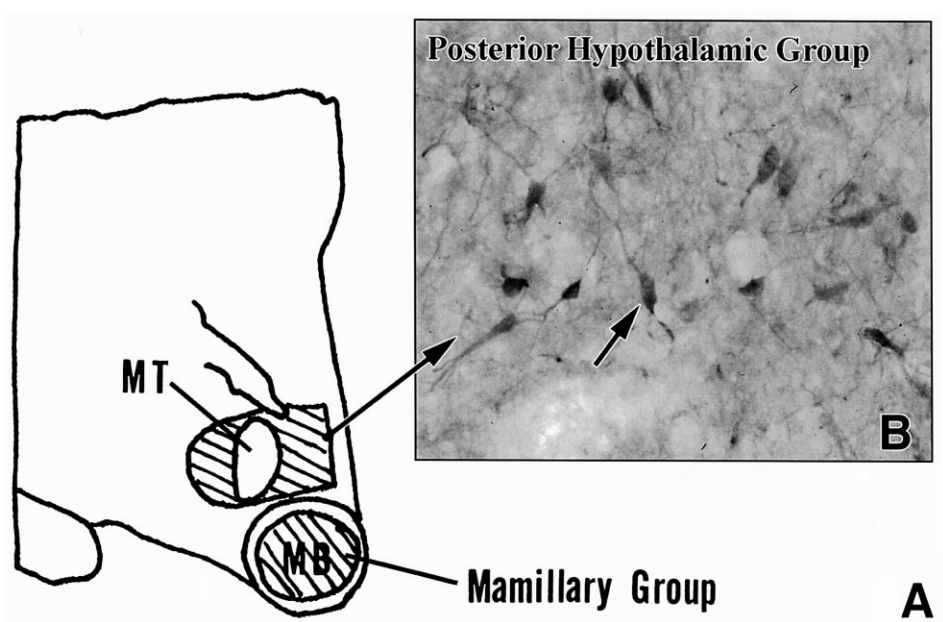

Fig. 5. (A) Schematic diagram showing ERIR neurons present in the posterior hypothalamic and mamillary groups (diagonal cross-hatching). MB = mamillary body, MT = mamillothalamic tract. (B) Photomicrograph showing neurons of the posterior hypothalamic group (arrow); the mamillary group looked very similar. They are smaller than the neurons of the nucleus basalis and about the same size as those of the lateral hypothalamic and infundibular groups. The staining of the neuronal nucleus, cytoplasm and processes is similar to that in other groups. ×200.

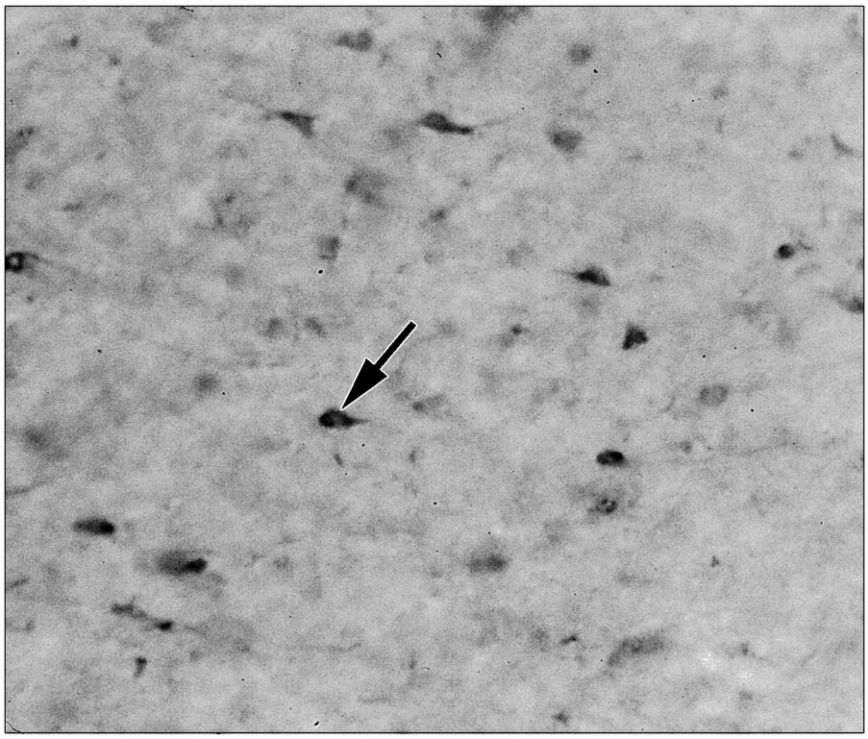

Fig. 6. Photomicrograph showing ERIR perikarya within the human amygdala. The strongest staining is in the area of the nucleus and surrounding cytoplasm (arrow). There is some staining of neuronal processes (axons and dendrites), but it is less intense and not well seen. These neurons tended to be smaller than those in the hypothalamus. ×200.

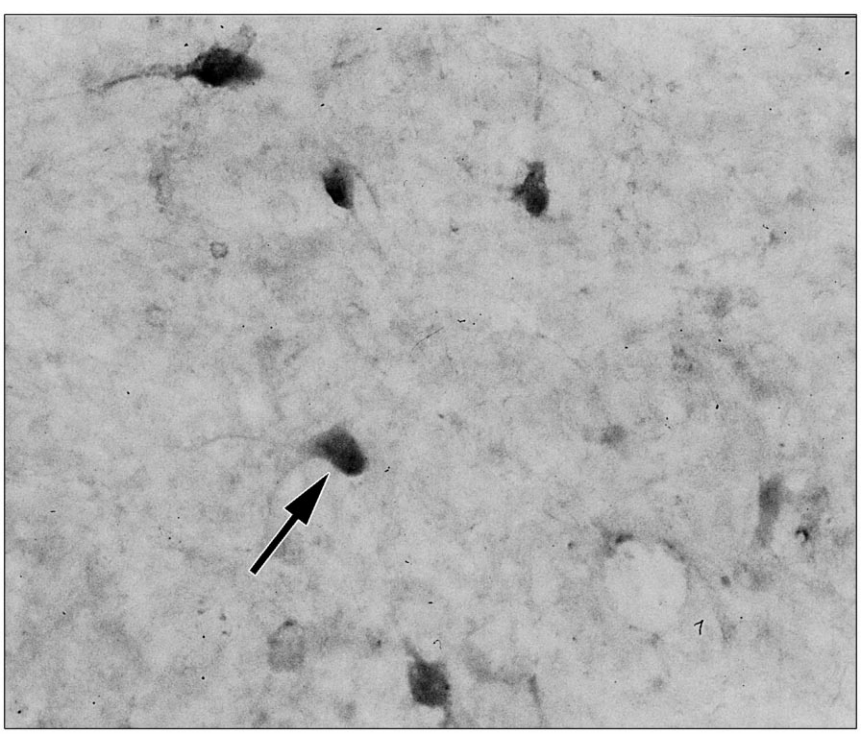

Fig. 7. Photomicrograph showing ERIR perikarya seen in the globus pallidus. Some of these neurons stained particularly intensely in the nucleus (arrow), surrounding cytoplasm of the cell body, and processes. These neurons were similar in size to those of the hypothalamus and larger than those of the amygdala. ×200.

dala. Their processes were better visualized than those of the amygdala.

4. Discussion

We report here the immunocytochemical identification of distinct subpopulations of ERIR neurons in the human preoptic area and infundibular regions in human brain. A predominantly ventromedial and periventricular group in the infundibular region, an anterior hypothalamic group in the preoptic area, a lateral hypothalamic group, and a posterior hypothalamic and mammillary group are found; these are illustrated schematically in Figs. 1–5. The spatial organization of these three groups is similar to that of ERIR neuronal subpopulations in rat [10,42,55,56], turtle [36], opossum [15,16], sheep [33], pig [65], monkey [24], ferret [57,59] and guinea pig brain [9,66]. Our findings support the hypothesis that these ERIR neurons are homologous to those seen in the preoptic area and infundibular region of the hypothalamus of other mammalian species.

In studies in several mammalian species, the ERIR neurons were found to be concentrated in the periventricular portion of the infundibular region and lateral regions of the preoptic area [9,34,36,42,46,66]. This has been shown by immunocytochemical localization of ERIR neurons in the guinea pig [9]. In our series, numerous ERIR neuronal cell bodies were seen in these similar regions, projecting to the third ventricle in the infundibular area and extending laterally in the preoptic area. The distribution of ERIR cells is consistent with the ER-α mRNA distribution obtained by Rance et al. [48] in human hypothalamus using in situ hybridization. In their study, ER-α mRNA-containing cells were more numerous than the ER-α protein-containing cells which we identified using immunocytochemistry. One might speculate, therefore, that there are a large number of cells that contain ER-α mRNA where the concentration of ER-α being expressed is below the level of detectability. Alternatively, the difference observed may also be explained by the greater sensitivity of in situ hybridization when compared to immunocytochemistry.

Our results confirm the existence of ERIR neurons in the amygdala and globus pallidus. The findings in the amygdala are consistent with those found in other species [5,14,33,34,57,59]. Studies in other species have also indicated that estrogens act directly in the striatum to affect behavior and dopamine receptors [3,26,49,68], and ER-α mRNA translation and ERIR cells have been detected in the basal ganglia of the developing brain using radiolabeling [53] and in situ hybridization [62]. However, classical estrogen receptors were not abundant in this region in vertebrates [68], and such was also the case in humans. A study by Tranque et al. [63] demonstrated an increased surface density of material immunoreactive to glial fibrillary acidic protein (GFAP) in the globus pallidus (and hippocampus) of estradiol-treated rats, suggesting a possible influence of estradiol on GFAP-immunoreactive glial processes. Whether this represents a direct effect of estrogens on astrocytes or an indirect influence mediated by neuronal–glial interactions remains to be elucidated.

The only other populations of cells that were found to be specifically ERIR were astrocyte-like cells and their processes in the lamina terminalis and subependymal region beneath the third ventricle (as seen schematically in Fig. 1), and some of the ependymal cells lining the ventricle, which likely correspond to the tanycytes. The subependymal cells exhibited prominent astrocytic characteristics such as a pale nucleus, fine chromatin staining, no nucleoli and numerous processes radiating from the cell body (Fig. 1B). The subependymal cells are thus most likely astrocytes morphologically. ER-α mRNA has been documented in glial cells grown in culture [50], which is consistent with our findings. The role of estrogen in these supporting glial cells remains unclear. In previous animal studies, high levels of endogenous estrogen produced numerous blebs and microvilli in tanycytes, which disappeared at low estrogen levels or after ovariectomy. However, these blebs and microvilli reappeared with the administration of estradiol benzoate [31]. Similar observations were subsequently made in the monkey [11], ewe [12,13] and hamster [37]. In adult rats, estrogens have also been shown to elicit a heat shock response and the accumulation of peroxidase-positive cytoplasmic inclusions in astrocytes of the hypothalamic arcuate nucleus and other ERIR brain regions [41]. Glial cells appear to be influenced by gonadal steroids [17,63], concentrate estrogen [43,45,61] and participate in the sexual differentiation of the brain [18,19,40,60]. This sexual differentiation, in which both neurons and glia evidently participate, leads to sex-specific changes such as cyclical leutinizing hormone (LH) secretion in females and a larger sexually dimorphic nucleus of the preoptic area in males [27].

In some of the cellular groups described above, particularly those where the size of the cells was relatively small, the ER-α reaction product within the cytoplasm approached that of the nucleus. Some of this increased cytoplasmic reactivity may be attributed to the rather high concentration of Triton x-100 used in our immunocytochemical protocol. Despite this, it is not unusual to find significant non-nuclear reactivity to ER-α in other species [5,6,59]. In addition, cells within the tissue adjacent to regions of positive reactivity are ERIR-negative, making non-specific labeling unlikely.

None of the ERIR neurons exhibited the bipolar characteristics of hypothalamic gonadotropin-releasing hormone (GnRH) neurons [28]. This suggests that the influence of estrogens on gonadotropin secretion is mediated through interneurons, rather than a direct effect on GnRH neurons. It is also consistent with the findings of Rance et al. [48] that ER-α mRNA-containing neurons do not contain GnRH mRNA. Alternatively, it may require a significantly more sensitive technique to pick up a small number of estrogen

receptors that GnRH neurons might or might not synthesize. Additionally, since we did not double-label for GnRH, it might be particularly difficult to detect likely GnRH neurons if it were only a small subgroup as was shown in guinea pigs [29].

The ERIR cells which we have identified are very likely to be involved in estrogen-mediated actions in the human brain. Estrogens induce choline acetyltransferase activity (the rate-limiting step in acetylcholine formation) in the neurons of the basal forebrain (nucleus basalis of Meynert) [39]; numbers of cholinergic neurons and acetylcholine levels are diminished in the forebrains of patients with Alzheimer's disease (AD) [67]. This could explain the efficacy of estrogen replacement therapy in the improvement of cognitive function in patients with AD [25,39,52,54]. Estrogens have also been shown to enhance transport of serotonin into neurons [39], which has clinical implications for depressive illness and migraine. Sex hormone fluctuations also greatly impact on the clinical expression of migraine [51]. Both of these disorders are associated with a serotonin deficiency state which may be amenable to estrogen replacement therapy [35,52].

In conclusion, we have demonstrated that neurons containing ER-α are distributed widely throughout the human hypothalamus and adjacent tissues. These results are in agreement with multiple studies in other animal species [9,10,15,16,24,33,36,42,55–57,59] and with the localization of ER-α mRNA within neurons of the human hypothalamus by in situ hybridization [48]. We have also shown that periventricular, subependymal astrocyte-like cells express ER-α. Non-reproductive functions of estrogens within the central nervous system are being elucidated which could lead to the increased use of estrogens as treatment for or prophylaxis against various neurologic and psychiatric conditions.

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