Age-related changes in the expression of ER- β mRNA in the female rat brain

Naoko Yamaguchi-Shima¹ and Kazunari Yuri*

Department of Neurobiology and Anatomy, Kochi Medical School, Kochi University,
Kohasu, Oko-cho, Nankoku, Kochi 783-8505, Japan

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* Corresponding author:

Kazunari Yuri

Department of Neurobiology and Anatomy

Kochi Medical School

Kochi University

Kohasu, Oko-cho, Nankoku

Kochi 783-8505, Japan

E-mail: yurik@kochi-u.ac.jp

Tel.: + 81-88-880-2297

Fax: + 81-88-880-2300

¹ Present address: Department of Pharmacology, Kochi Medical School, Kochi University, Kohasu, Oko-cho, Nankoku, Kochi 783-8505, Japan.

Abstract

Estrogen is important for numerous physiological actions, most of which are mediated via the nuclear estrogen receptors (ERs), ER- α and ER- β , which modulate the transcription of target genes following estrogen binding. Estrogen functions change with age. In the present study, to reveal the effects of normal aging on ER- β expression in the brain, we examined ER- β expression at the transcriptional level using young (10 weeks), middle-aged (12 months) and old (24 months) intact female rats. In situ hybridization using a digoxigenin-labeled RNA probe was used to assess the number of ER- β mRNA-positive cells in each region in whole brain. ER- β mRNA-positive cells were detected throughout the brain in young female rats and were reduced in number in the olfactory bulb, cerebral cortex, hippocampus, accumbens nucleus, part of the amygdala and raphe nucleus of middle-aged rats, but did not decline further in number in aged animals. By contrast, the number of ER- β mRNA-positive cells was decreased in the hippocampus, caudate putamen, claustrum, accumbens nucleus, substantia nigra and cerebellum was not significantly different between young and middle-aged rats, but was decreased in old rats. These results indicate that the expression of ER- β mRNA in the female rat brain is differentially modulated during aging and that the changes are region specific.

Section: 2) Nervous System Development, Regeneration and Aging

Keywords: aging; estrogen; estrogen receptor; brain.

Abbreviations: ER, estrogen receptor.

1. Introduction

Estrogen exerts various effects on its target tissues, such as those of the reproductive, skeletal and cardiovascular systems. In the central nervous system, estrogen plays important roles not only in the control of reproductive actions, such as sex differentiation, but also in the regulation of a variety of nonreproductive functions, such as neuroprotection (Dluzen, 2000; Garcia-Segura et al., 2001; Behl, 2002; McEwen, 2002), synaptogenesis (Desmond and Levy, 1997; McEwen et al., 2001; Adams et al., 2001), cognition (Sherwin, 1994; Tang et al., 1996; McEwen et al., 1997; Sandstrom and Williams, 2001; Rissman et al., 2002) and fine motor skills or movement (Hampson, 1990; Jennings et al., 1998).

The actions of estrogen are mediated mainly through its receptors (ERs) that modulate the transcription of their target genes as either homodimers or heterodimers following estrogen binding. The ER is a member of the nuclear receptor superfamily and has two isoforms, ER- α and ER- β . ER- β , which was cloned from rat prostate (Kuiper et al., 1996), is highly homologous to ER- α .

In the rat brain, ER- β mRNA is distributed widely, in a pattern different from that of ER- α mRNA (Shughrue et al., 1997; Shima et al., 2003). Both ER- α and ER- β mRNA are abundant in the same brain regions: the preoptic area, bed nucleus of the stria terminalis and medial amygdala (Simerly et al., 1990; Shughrue et al., 1997). ER- β mRNA was shown to be co-expressed with ER- α immunoreactivity in the same neuron in several brain regions: the bed nucleus of the stria terminalis, medial amygdaloid nucleus, periventricular preoptic nucleus, medial preoptic nucleus, and anteroventral periventricular nucleus (Shughrue et al., 1998; Orikasa et al., 2002). In addition, in vitro studies have shown that ER- β can form heterodimers with ER- α (Cowley et al., 1997; Pace et al., 1997; Pettersson et al., 1997; Ogawa et al., 1998; Matsuda et al., 2002),

suggesting that estrogen may differentially modulate the activity of certain neuronal populations depending on whether the cells express ER- α , ER- β or both (Shughrue et al., 1998). ER- β mRNA is also highly distributed in several rat brain regions, including the olfactory bulb, cerebral cortex, paraventricular hypothalamic nucleus, supraoptic nucleus, hippocampus and cerebellar Purkinje cells, areas that contain little or no ER- α mRNA (Simerly et al., 1990; Shughrue et al., 1997; Mufson et al., 1999; Shima et al., 2003; Mehra et al., 2005). The differences in the distribution patterns of ER- α and ER- β suggest different physiological roles of each receptor subtype in the central nervous system.

The age-related changes in circulating estrogen have broad consequences on the body and brain (Chakraborty and Gore, 2004). During aging in female rats, estrogen decreases are associated with a loss of reproductive cyclicity that appears to result primarily from hypothalamic dysfunction (Miller et al., 1994). In addition to changes in reproductive function, it is known that loss of estrogen with age affects structural and functional changes in vasomotor reactions, depressed mood and cognitive impairment (Lamberts et al., 1997; Chakraborty and Gore, 2004; Thakur and Sharma, 2006).

Sensitivity to estrogen is also reduced with aging, which might be correlated with a decline in the number of ERs with age. Many studies that compared aged female rats with young rats have revealed a decrease in cell nuclear estrogen-binding capacities in the brain, especially in the hypothalamus (Kanungo et al., 1975; Wise and Camp, 1984; Wise and Parsons, 1984; Wise et al., 1984; Rubin et al., 1986; Brown et al., 1990). Haji et al. (1981) have shown that the $B_{\max}$ of cytosolic ERs in the hypothalamus decreased in middle-aged and old female rats compared with that of young rats, but no change with age was apparent in ER binding properties, suggesting that the age-associated reduction in $B_{\max}$ may be due to a reduction in the number of binding sites. These data indicate that decreases in maximal nuclear ER concentrations may be the result of a decreased number

of cytoplasmic ERs available for translocation and not to changes in the subcellular distribution of the receptor in aging rats (Wise et al., 1984). While previous studies showed that ER- α expression changes during aging in the boundaries of the medial septal-vertical limb of the diagonal band of Broca, periventricular preoptic nucleus, ventromedial nucleus and hippocampus in the female rat brain (Funabashi et al., 2000; Adams et al., 2002; Wilson et al., 2002; Chakraborty et al., 2003a; Kalesnykas et al., 2005; Mehra et al., 2005), little is known about the effect of aging on the level of ER- β expression.

Although it is well known that the circulating level of estrogen decreases as a part of the normal aging process in women and female rats (Lu et al., 1981; Lamberts et al., 1997) and that the actions of estrogen change with age, the effects of aging on ER- β that play an important role in mediating estrogenic actions are poorly understood. Therefore, in the present study, we analyzed age-related changes in ER- β expression in the female rat brain at the transcriptional level under the physiological conditions.

2. Results

As expected, hybridization signals for ER- β were detected throughout the extent of the brain in young female rats (Table 1). Although there was no difference among age groups in the brain regions in which ER- β mRNA-positive cells were observed, the number of ER- β mRNA-positive cells changed with age in specific brain regions. The antisense probe detected ER- β in the cytoplasm of neurons, but not their nuclei. Sections incubated with the sense probe for ER- β mRNA or treated with RNase A before hybridization using the antisense probe showed no hybridization signal in any brain region (data not shown).

In the telencephalon, intense hybridization signals for ER- β mRNA were observed in the allocortex (piriform and entorhinal cortex), indusium griseum and amygdala, especially in the central and medial subnuclei, across all age groups (Table 1). Moderate signals were observed in the olfactory tubercle, isocortex, endopiriform nucleus, septum, diagonal band of Broca, bed nucleus of the stria terminalis and amygdala, except for the central, medial and basolateral subnuclei (Table 1). In these brain regions, no age-related changes in ER- β mRNA expression were detected (Table 1). Age-related changes were, however detected in a number of other areas. ER- β mRNA-positive cells were detected with abundance in the mitral cell layer (Fig. 1A) and anterior olfactory nucleus in the olfactory bulb, layer 6 of the cortex, basolateral nucleus in the amygdala and nucleus of the lateral olfactory tract in young rats. The levels of ER- β mRNA detected in these areas were decreased significantly in middle-aged rats compared to young rats, whereas no age-related difference was found between the middle-aged and old rats (Fig. 1A-C, 2). In the caudate putamen and claustrum, there were no significant differences between young and middle-aged rats, whereas a significant decline in ER- β mRNA levels was found in these regions between middle-aged and old rats (Table 1, Fig. 2). In the CA1 to

CA3 pyramidal layers of the hippocampus and accumbens nucleus, unlike other brain regions in the telencephalon, ER- β mRNA-positive cells were present in moderate-to-high levels in young rats and decreased in number gradually with age; significant differences were found among the three age groups (Fig. 1D-I, 2).

In the diencephalon, ER- β mRNA-positive cells were abundant in the paraventricular hypothalamic nucleus, supraoptic nucleus and ventromedial hypothalamic nucleus in all three age groups (Table 1). Moderate signals were detected in the thalamic nuclei, preoptic area, periventricular nucleus, suprachiasmatic nucleus and arcuate nucleus (Table 1). In these brain regions, the levels of ER- β mRNA expression did not differ with age (Table 1).

In the mesencephalon, ER- β mRNA-positive cells were detected in several regions, such as the red nucleus, oculomotor nucleus and mesencephalic trigeminal nucleus, and the levels of ER- β mRNA expression did not differ among age groups (Table 1). In the substantia nigra, ER- β mRNA-positive cells were detected at a moderate level, and a significant decrease in ER- β mRNA was found in the reticular part between the old and middle-aged rats (Fig. 2), but not the compact part. In the raphe magnus nucleus, low-to-moderate hybridization signals detected in the young rats were also reduced with age (Fig. 1J-L, 2). In the metencephalon, ER- β mRNA was highly expressed in the locus coeruleus, and the expression level in this nucleus did not change with age (Table 1). In the cerebellar Purkinje cells, moderate hybridization signals detected in the young and middle-aged rats were decreased significantly in the old rats (Fig. 1M-O, 2).

3. Discussion

Estrogen exhibits various effects in the body and brain via its receptors, ER- α and ER- β , and the actions of this hormone change during aging. In the present study, we showed the effect of aging on ER- β mRNA expression in the whole brain of intact female rats. Clear signals were obtained for ER- β mRNA in the cytoplasm, but not in the nucleus, which is consistent with localization to the endoplasmic reticulum, the site of protein production (Pasterkamp et al., 1997).

In this study, ER- β mRNA was distributed widely in the brain of young female rats sacrificed in their diestrus stage, in a pattern that was nearly consistent with that reported in previous studies that used ovariectomized rats. Similar to those studies (Shughrue et al., 1996, 1997, 1998; Hrabovszky et al., 1998; Price Jr. and Handa, 2000; Shima et al., 2003), we detected ER- β mRNA with abundance in brain areas including the olfactory bulb, hippocampus, amygdala, and supraoptic and paraventricular nuclei of the hypothalamus.

Wilson et al. (2002) examined the effects of aging on ER- β mRNA expression in the cortex, preoptic area, paraventricular hypothalamic nucleus and supraoptic nucleus, and detected decreases in only the cortex and supraoptic nucleus in ovariectomized aged rats compared with young rats. The present findings were in accordance with their previous results. In addition to brain regions reported previously, we revealed the age-related decreases in the ER- β mRNA expression in the olfactory bulb, caudate putamen, claustrum, accumbens nucleus, part of the amygdala, substantia nigra, raphe magnus nucleus and cerebellar Purkinje cells in the present study. Our results provide new information about the effect of aging on ER- β mRNA expression in the whole brain of intact female rats. The age-related decreases in the level of ER- β protein have reported in the cortex of the intact and ovariectomized female mice (Sharma et al., 2006) and in

the hippocampus of female rats (Mehra et al., 2005), which is in agreement with our and previous data for ER- β mRNA. Chakraborty et al. (2003b) reported that ER- β immunoreactive cells decreased with age in the anteroventral periventricular nucleus but not the bed nucleus of the stria terminalis. By contrast, we found no changes in ER- β mRNA levels in either brain region. Although the cause of this discrepancy is not clear, there is a possibility that the age-related decrease in ER- β occurs at the translational level of the ER gene in part of the anteroventral periventricular nucleus.

It is unclear whether the age-related decline in ER- β mRNA expression showed in previous and this studies might be due to reduction in cell number or less expression of the mRNA per cell. In the hippocampus, it has been demonstrated that the number of neurons was preserved in the aged rats, indicating that the hippocampal neuronal degeneration is not an inevitable consequence of normal aging (Rapp and Gallagher, 1996). Similarly, Mehra et al. (2005) indicated that the reduction in the number of ER- β positive neurons during aging is a consequence of antigen loss rather than of the actual loss of neurons by degeneration.

The present results suggest that the actions of estrogen in aged animals can be affected by the loss of receptors in specific brain regions, resulting in a decreased binding capacity of and diminished responsiveness to estrogen. Since estrogen bound to its receptors translocates into the nucleus and modulates the transcription of target genes, the action of estrogen appears to be affected by the concentrations of ERs in the target cells, namely the amount of estrogen-ER complex. It is possible that the decreased ER- β concentration influences the total number of available receptors, leading to a diminished responsiveness to estrogen. Moreover, it has been reported that the ER- α protein and the ER- β mRNA are co-localized in neurons in the rat brain (Shughrue et al., 1998; Orikasa et al., 2002), and the activity of estrogenic compounds may depend, in part, on whether a cell contains ER- α , β , or both. In addition, it is known that ERs form homo- or

heterodimers (Cowley et al., 1997; Pace et al., 1997; Pettersson et al., 1997; Ogawa et al., 1998; Matsuda et al., 2002). There is a possibility that estrogen demonstrates different physiological actions via homo- or heterodimers, and the formation of dimers may be affected by decreased levels of ER- β in the specific brain regions in which decreases were observed in aged female rats.

As a result of age-related decreases in ER- β expression, estrogen-dependent functions may change in older animals. For instance, many reports have indicated a relationship between estrogen and cognitive function in humans and rodents (Warren and Juraska, 1997; Luine et al., 1998; Markowska, 1999; Fugger et al., 2000; Heikkinen et al., 2002; Iivonen et al., 2006). In postmenopausal women and aged rodents, estrogen treatment enhances cognitive function (Drake et al., 2000; Keenan et al., 2001; Sherwin, 2002; Markowska and Savonenko, 2002; Frick et al., 2002). The hormonal influences on cognitive processes appear to involve actions on the hippocampus and basal forebrain (McEwen and Alves, 1999). ER- β mRNA is distributed in hippocampal and cerebral cortical regions that contain little or no ER- α (Shughrue et al., 1997; Li et al., 1997; Shima et al., 2003), implying the possibility that estrogen is involved in cognitive function through activating ER- β . Moreover, the decline in receptor expression may correlate with the cognitive decline seen in aged humans and rodents (Wilson et al., 2002). In this study, we showed an age-related decline in cortical and hippocampal ER- β expression, which corresponds to results of previous studies (Wilson et al., 2002; Mehra et al., 2005; Sharma et al., 2006). These data suggest that the age-related reduction of the ER- β mRNA level in these regions may be associated with the changes in cognitive function in aged female rats.

In contrast to the changes in the hippocampus and cortex, the number of ER- β mRNA-positive cells was not affected with aging in the preoptic area, anteroventral periventricular nucleus, suprachiasmatic nucleus and ventromedial hypothalamic nucleus,

areas known to be involved with the control of reproductive function and behavior. In contrast, many studies have shown a reduction in ER- α mRNA in ovariectomized rats with age in such brain regions (Wise and Parsons, 1984; Wise et al., 1984; Funabashi et al., 2000). These data suggest the importance of ER- α , but not ER- β , in reproductive functions regulated by estrogen, which supports the results of other studies showing the role of ERs in reproductive function using ER-knockout mice. ER- α knockout male and female mice showed various abnormalities in reproductive aspects, including infertility, no sign of lordosis, and infanticide (Lubahn et al., 1993; Ogawa et al., 1996; Ogawa et al., 1998; Simpson, 1998; Rissman et al., 1999). In contrast, the analyses of reproductive behaviors of ER- β knockout mice indicated the possibility that ER- β is not essential for such behaviors (Krege et al., 1998), since ER- β knockout male and female mice exhibited normal reproductive behaviors (Krege et al., 1998; Ogawa et al., 1999). Taken together, these findings suggest that ER- α , but not ER- β , may be critical to age-related reductions in reproductive function in aged rats.

In summary, we have demonstrated that the number of ER- β mRNA-positive cells decreased during aging in the female rat brain in a region-specific manner, suggesting the possibility that diminished ER- β mRNA expression is involved in the age-related changes of estrogen-dependent functions in these brain regions.

4. Experimental procedures

4. 1. Animals

Young (10 weeks, n = 6), middle-aged (12 months, n = 6) and old (24 months, n = 4) female Wistar/ST rats (body weight 210-225 g, 260-300 g and 395-420 g, respectively) were used. Rats were purchased from SLC (Shizuoka, Japan) and maintained in our animal facilities under a controlled light and temperature environment (14:10 light:dark cycle, 23°C), with food and water provided ad libitum. All rats were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH, 1996) and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Kochi Medical School, Kochi University.

For all rats, estrous cycle stage was checked by vaginal cytology at 8:30 a.m. All young rats exhibited at least two regular 4-day estrous cycles, all middle-aged rats exhibited irregular cycles and all old rats were in constant diestrus. All of the animals were sacrificed in diestrus. The animals were anesthetized in the morning with a mixture of 20 mg/ml tribromoethanol, 80 µl/ml ethanol, 12 µl/ml amylalcohol and 9 mg/ml NaCl (1.0 ml/100 g body weight, i.p.), and perfused through the left cardiac ventricle with 100 ml phosphate-buffered saline (pH 7.4) followed by 500 ml ice-cold fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed, post-fixed in the same fixative for 24 hours and cryoprotected in 20% sucrose in 0.1 M phosphate buffer at 4°C. Coronal frozen sections (14 µm) were cut on a cryostat, thaw-mounted on silane-coated slides and then stored at -80°C.

4. 2. In situ hybridization

Preparation of probes and in situ hybridization were performed as described previously (Shima et al., 2003). Briefly, a 391-bp (bases 1-391) ER-β complementary

RNA probe corresponding to an EcoRI-AccI fragment of ER- β was used for in situ hybridization (Kuiper et al., 1996). For transcription, linearized DNA was incubated in transcription buffer containing digoxigenin (DIG)-UTP. The concentration of the resulting RNA probe was estimated to be 7.5 to 12.5 $\mu\text{g}/50\text{ }\mu\text{l}$ by electrophoresis. The ER- β RNA probe used in this study had less than 20% amino acid homology to ER- α .

The brain sections on the slides were fixed in 4% paraformaldehyde solution for 15 minutes. The sections were treated with proteinase K (20 $\mu\text{g}/\text{ml}$) in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) for 10 minutes at 37°C, 0.25% acetic anhydride in 0.1 M triethanolamine-HCl pH 8.0 for 10 minutes and dehydrated through an ascending alcohol series at room temperature. Hybridization buffer (50% formamide, 1x SET, 0.25% SDS, 200 $\mu\text{g}/\text{ml}$ tRNA, 1x Denhardt's solution, 10% dextran sulfate) containing 500 ng antisense or sense probe per ml was applied to each slide (100 $\mu\text{l}/\text{slide}$), and hybridization was carried out for 18 hours at 55°C. Following hybridization, the slides were washed in 2x SSC containing 50% formamide for 30 minutes at 55°C, treated with RNase A (12.5 $\mu\text{g}/\text{ml}$) in TNE buffer (10 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM EDTA) for 30 minutes at 37°C and washed in 2x SSC twice for 20 minutes and 0.2x SSC for 20 minutes at 55°C. Following a wash in buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl) and incubation in blocking solution for 1 hour, the slides were incubated with anti-DIG-AP Fab fragment (1:500, Boehringer, Mannheim, Germany) for 30 minutes at room temperature. They were washed in buffer 1 and buffer 3 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl_2), and immuno-detection was carried out in AP-substrate solution (NBT/BCIP) for 15 hours at room temperature.

4. 3. Data analysis

Adjacent sections were Nissl-stained with cresyl violet, and the boundary of the nucleus was determined. The classification of the nuclei was according to the atlas of

Paxinos and Watson (1998). The distribution and density of ER- β mRNA-positive cells were observed and compared among age groups. The relative densities were rated according to the following criteria: (-) if no labeled cells were observed, low (+) if less than 30% of the cells were labeled, moderate (++) if 30%-70% of the cells were labeled and high (+++) if more than 70% of the cells were labeled. In the brain regions observed, to determine the differences in the number of ER- β mRNA-positive cells among age groups, the number of ER- β mRNA-positive cells was counted in 5 grids per rat in each brain area. A grid (grid area: $150 \times 200 = 30,000 \mu\text{m}^2$, except for the cerebral cortex and cerebellum; $100 \times 300 = 30,000 \mu\text{m}^2$) was placed in a section in each region, and the mean in 5 grids was assessed. Data were analyzed by one-way analysis of variance (ANOVA), followed by post-hoc analysis with the Bonferroni method, and the differences were considered to be significant if the p value was less than 0.05.

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Figure legends

Fig. 1. Photomicrographs of ER- β mRNA-positive cells in the mitral cell layer of the olfactory bulb (A, B, C), hippocampus (D, E, F), accumbens nucleus (G, H, I), raphe magnus nucleus (J, K, L), and cerebellar Purkinje cells (M, N, O) of young (10 weeks, A, D, G, J, M), middle-aged (12 months, B, E, H, K, N) and old (24 months, C, F, I, L, O) rats. Each is a representative region in which the number of ER- β mRNA-positive cells was decreased significantly with age. Scale bars = 50 μ m.

Fig. 2. Quantification of ER- β mRNA-positive cells in young (10 weeks), middle-aged (12 months) and old (24 months) rats. ER- β mRNA-positive cells were reduced significantly with age in specific brain regions. Results represent the means $\pm$ S.E.M. *Significantly different ($p < 0.05$) from the young group with the Bonferroni method. #Significantly different ($p < 0.05$) from the middle-aged group with the Bonferroni method. Acb, accumbens nucleus; AON, anterior olfactory nucleus in the olfactory bulb; BL, basolateral nucleus in the amygdala; Cl, claustrum; CPu, caudate putamen; LOT, nucleus of the lateral olfactory tract; Mi, mitral cell layer of the olfactory bulb; RMg, raphe magnus nucleus; SNR, the reticular part of the substantia nigra.

Table. 1. Relative densities of estrogen receptor- β mRNA-positive cells in the brains of young (10 weeks), middle-aged (12 months) and old (24 months) female rats.

Region	Young	MA	Old
<i>Telencephalon</i>			
Olfactory bulb			
Mitral cell layer of olfactory bulb	++	+	+
Anterior olfactory nucleus	+++	++	++
Olfactory tubercle	++	++	++
Allocortex			
Piriform cortex	+++	+++	+++
Entorhinal cortex	+++	+++	+++
Isocortex			
Layers 2-3	++	++	++
Layers 4-5	++	++	++
Layer 6	++	+	+
Indusium griseum	+++	+++	+++
Hippocampal formation			
CA1-CA3	+++	+++	++
Caudate putamen	+	+	+/-
Clastrum	+++	+++	++

Endopiriform nucleus				
	Dorsal	++	++	+
	Ventral	+	+	+/-
Accumbens nucleus				
		++	+	+/-
Septum				
	Septohippocampal nucleus	++	++	+
	Medial septal nucleus	++	++	+
	Lateral septal nucleus			
	Dorsal part	++	++	+
	Intermediate	+	+	+/-
Diagonal band of Broca				
	Horizontal limb	++	++	++
	Vertical limb	++	++	+
Bed nucleus of the stria terminalis				
		++	++	++
Amygdala				
	Amygdalohippocampal area	++	++	++
	Central nucleus	+++	+++	+++
Cortical nuclei				
	Anterior	++	++	++
	Postlateral	++	++	++
	Postmedial	++	++	++

Lateral nucleus	++	++	++
Medial nucleus	+++	+++	+++
Basolateral nucleus	+++	++	++
Basomedial nucleus	+	+	+
Anterior area	+	+	+
Amygdalopiriform transition area	++	++	++
Nucleus of the lateral olfactory tract	+++	++	++
Subfornical organ	++	++	++

Diencephalon

Thalamus			
Anterodorsal nucleus	++	++	++
Anteroventral nucleus	++	++	+
Anteromedial nucleus	++	++	++
Central medial nucleus	++	++	++
Laterodorsal nucleus	++	++	++
Lateral posterior nucleus	++	++	++
Ventral anterior nucleus	++	++	++
Ventrolateral nucleus	++	++	+
Ventromedial nucleus	++	++	++
Ventral posterior nucleus	++	++	++
Paraventricular nucleus	++	++	++

Reticular nucleus	+	+	+
Zona incerta	+	+	+
Habenula			
Medial habenular nucleus	++	++	++
Lateral habenular nucleus	+/-	+/-	+/-
Hypothalamus			
Preoptic area			
Medial	++	++	++
Ventromedial nucleus	++	++	++
Magnocellular nucleus	++	++	++
Anterior hypothalamic area	+	+	+
Posterior hypothalamic area	++	++	++
Lateral hypothalamic area	+/-	+/-	+/-
Lateroanterior hypothalamic nucleus	++	++	++
Periventricular nucleus	++	++	+
Anteroventral periventricular nucleus	++	++	++
Paraventricular nucleus	+++	+++	+++
Suprachiasmatic nucleus	++	++	++
Supraoptic nucleus	+++	+++	+++
Arcuate nucleus	++	++	++
Ventromedial hypothalamic nucleus	+++	+++	+++

Mesencephalon

Interpeduncular nucleus	++	++	++
Substantia nigra			
Compact part	++	++	++
Reticular part	++	++	+
Red nucleus	++	++	++
Oculomotor nucleus	++	++	++
Mesencephalic trigeminal nucleus	++	++	++
Raphe			
Raphe magnus nucleus	+	+/-	+/-

Metencephalon

Cerebellum			
Purkinje cells	++	++	+
Locus coeruleus	+++	+++	+++
Motor trigeminal nucleus	++	++	++
Principal sensory trigeminal nucleus	++	++	++
Nucleus of the trapezoid body	++	++	++
Pontine nuclei	++	++	++
Superior olive	++	++	++

Relative density: -, none; +-, very low; +, low; ++, moderate; +++, high. MA, middle-aged rats.