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ORIGINAL PAPER

Aging-induced changes in sex-steroidogenic enzymes and sex-steroid receptors in the cortex, hypothalamus and cerebellum

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Abstract We investigated age-induced changes in mRNA expression profiles of sex-steroidogenic enzymes and sex-steroid receptors in 3-, 12-, and 24-month-old male rat brain subregions [cerebral cortex (CC), hypothalamus (Hy) and cerebellum (CL)]. In many cases, the expression levels of mRNA decreased with age for androgen synthesis enzyme systems, including *Cyp17a1*, *Hsd17b* and *Srd5a* in the CC and CL, but not in the Hy. Estradiol synthase *Cyp19a1* did not show age-induced decline in the Hy, and nearly no expression of *Cyp19a1* was observed in the CC and CL over 3–24 m. Androgen receptor *Ar* increased in the Hy but decreased in the CC with age. Estrogen receptor *Esr1* increased in the CC and Hy, and did not change in the CL with age. *Esr2* did not change in the CC and Hy, but decreased in the CL with age. As a comparison, age-induced changes of brain-derived neurotrophic factor mRNA were also investigated.

Keywords Aging · Brain · Cortex · Hypothalamus · Cerebellum · Steroidogenesis · Steroid receptor

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Introduction

Many studies have been accumulated for aging effects on the cognitive impairment or dysfunction of the reproductive system [2, 17]. A decline in sex steroids, especially 17β-estradiol (E2), has been thought to be one of the most important factors involved in age-related neural dysfunction, since E2 significantly decreases in plasma upon menopause, which elicits cognitive decline and dysfunction of the hypothalamus–pituitary–gonadal axis [31]. The relationship between neural function and E2 has been extensively investigated, including the modulatory effect of E2 on synaptic plasticity [4, 18, 21], and the molecular mechanism of the beneficial effect of E2 replacement therapy by using rodents and primates [6, 25]. Recently, studies on the relationship between androgen decline and age-related neural and reproductive dysfunction have also been performed in male mice [22].

These aging effects had been attributed to the decline in plasma E2 and testosterone (T), which are synthesized in the ovary and testis [10, 31]. Importantly, recent studies demonstrate that young adult male and female rat hippocampal neurons synthesize estrogen and androgen whose levels are higher than those in the plasma (Fig. S1) [7, 9, 11, 13, 15]. Therefore, age-related changes in brain sex-

steroid synthesis should be investigated, since brain derived sex steroids may be more effective than gonadal sex steroids on synaptic plasticity with age.

Here, we investigated age-induced changes in the mRNA level of steroidogenic enzymes and steroid receptors in the cerebral cortex (CC), hypothalamus (Hy), and cerebellum (CL), in which endogenous steroidogenesis has not been extensively investigated in adult and aged animals. We compared young adult (3-month-old, 3 m), middle-aged (12-month-old, 12 m) and aged (24-month-old, 24 m) male rats. We observed that aging induced not only monotonous decrease but also increase (often in Hy), and no change in mRNAs, depending on individual mRNAs and difference in regions.

Materials and methods

Animals

Male Wistar rats of 3, 12 and 24 m were used in the current study. We used normal aged rats in which the brains did not have tumor-like structures including abnormal pituitary and hypophyseal adenomas. Animals were housed in the animal facility of the Tokyo Metropolitan Institute of Gerontology, under a 12 h-light/dark cycle and were allowed ad libitum access to food and water. All experiments were performed with the approval of the Committee for Animal Research of the University of Tokyo.

Total RNA isolation

Rats were deeply anesthetized and were decapitated. The CC, Hy, CL and hippocampus (Hi) were quickly removed and immersed in ice-cold artificial cerebrospinal fluid (pH 7.4, 290 mOsm), and stored in liquid nitrogen until use. Total RNA was extracted using an SV Total RNA Isolation System (Promega, USA) according to the manufacturer's instructions. Total RNA was then treated with Recombinant DNase I (RNase-free DNase I; Takara, Japan), and then purified. The concentration was quantified by absorption at 260 and 280 nm.

Primer design

Since the expression of steroidogenic enzymes and steroid receptors is extremely low in the brain, PCR primers with high sensitivity and specificity were carefully designed for precise analysis (Table 1) [15]. We designed the primers considering Gibbs energy (ΔG) as follows: (1) ΔG of the whole primer [$\Delta G_{(\mathrm{whole})}$] was calculated with a nearest-neighbor model [1], and set to be below the mean value for all of the primer candidates (ΔG_{av}) to obtain good stability in primer-target interaction. (2) ΔG of the five 3′-terminal bases of the primer [$\Delta G_{(5\mathrm{base})}$] was set to be higher than ΔG_{av} for improved specificity. Consequently, ΔG of 5′-terminal bases was set to be less than the ΔG_{av}, resulting not only in the improved stability in 5′-terminal interaction but also in the improved DNA polymerase recognition. Two (forward and reverse) primers were designed on separate exons and primers were designed to avoid 'PCR debris' including primer dimers.

RT-PCR

RT-PCR was performed essentially as described elsewhere [15]. The sequences of oligonucleotide primers, the number of PCR cycles, and the PCR cycle numbers for exponential amplification phase, used in RT-PCR, are shown in Table 1. For RT, total RNA was reverse-transcribed into first-strand cDNA using an oligo(dT) primer. The reaction solution (25 μl) contained 10 μg of total RNA, 1 × RT buffer, 1 mM dNTP mixture, 2 μg oligo$(dT)_{15}$ (Promega, USA), 40 U RNasin *Plus* (Promega), and 200 U RTase (Toyobo, Japan). The reaction was carried out at 42 °C for 60 min, and stopped by heating at 75 °C for 15 min. cDNA was treated with 4 U RNase H (Takara bio, Japan) at 37 °C for 30 min, and stored at −20 °C until use.

PCR was performed in 25 μl of PCR mixture comprising cDNA corresponding to 100 ng of total RNA, 1 × PCR buffer, 0.2 M dNTP mixture, 0.2 μM forward and reverse primers, and 0.63 U Blend Taq polymerase (Toyobo, Japan). PCR was performed with cycle reactions at 95 °C for 30 s, 56–68 °C for 20 s, and 72 °C for 30 s, with an initial denaturing at 95 °C for 2 min and a final elongation at 72 °C for 5 min. The PCR products were applied to 2 % agarose gels. Gels were stained with ethidium bromide (EtBr) and visualized under UV light. Fluorescence images were recorded with Printgraph (ATTO, Japan). For quantitative estimation, images of the bands were analyzed using the Image J software (National Institutes of Health, Bethesda, MD). In all the cases, we first plotted amplification curves in order to obtain the exponential amplification phase of the PCR plot.

DNA sequencing

PCR products were extracted from agarose gels using a Wizard SV Gel and PCR Clean-up System (Promega) and cloned into pGEM-T-Easy vectors (Promega, USA). Sequencing reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). Signals were detected using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, USA). In all the expression analyses, the sequence identity

Table 1 Primers and PCR conditions

Enzyme/protein name	Gene name	Accession no.	Direction	Primer sequence (5′–3′)	Product length [bp]	T_a (°C)	PCR cycles	Exponential amplification
StAR	*Star*	NM_031558	Forward	5′-CTGGTGGGGCCCCGAGACTT-3′	360	60	32	28–34
			Reverse	5′-CAATGGCGTGCAGGTAGATGTGGT-3′				
TSPO	*Tspo*	NM_012515	Forward	5′-GGCTATGGTTCCCTTGGGTCTCTACAC-3′	362	63	28	26–30
			Reverse	5′-AGGCCCAATGGTCATGAAAGCAGGAT-3′				
P450(17α)	*Cyp17a1*	NM_012753	Forward	5′-TGGGGCGGGCATAGAGACAACT-3′	477	62	33	30–36
			Reverse	5′-AGCAAGGCCGTGAAGACAAAGAGC-3′				
17β-HSD1	*Hsd17b1*	NM_012851	Forward	5′-ACTCCGGGCGTGTGCTGGTGA-3′	517	65	30	28–32
			Reverse	5′-GGCGTGTCTGGATCCCCTGAAACTT-3′				
17β-HSD3	*Hsd17b3*	NM_054007	Forward	5′-CTCCCCAACCTGCTCCCAAGTCATTT-3′	408	65	31	29–33
			Reverse	5′-AGCAAGGCAGCCACAGGTTTCAGC-3′				
5α-reductase1	*Srd5a1*	NM_017070	Forward	5′-ACCGCGTCCTGCTGGCTATGTTT-3′	318	63	25	23–27
			Reverse	5′-GGCCTCCCCTGGGTATCTTGTATCC-3′				
5α-reductase2	*Srd5a2*	NM_022711	Forward	5′-AGGTGGCTTGTTTACGTATGTCTCTG-3′	453	57	32	29–35
			Reverse	5′-GGCCTCTGTGAAGCTCCAAAAG-3′				
P450arom	*Cyp19a1*	NM_017085	Forward	5′-CTGATCATGGGCCTCCTCCTG-3′	276	58	32	30–34
			Reverse	5′-CCCACGCTTGCTGCCGAATCT-3′				
AR	*Ar*	NM_012502	Forward	5′-CAACTTTCCGCTCGCTCTGTC-3′	536	56	26	24–28
			Reverse	5′-TCTGGGGTGGGAAGTAATAGTCG-3′				
ERα	*Esr1*	NM_012689	Forward	5′-GCCGGCTGCGCAAGTGTTACG-3′	467	68	28	26–30
			Reverse	5′-GGAGCGCCAGACCAGACCAATCA-3′				
ERβ	*Esr2*	NM_012754	Forward	5′-GCAAACCAGGAGGCAGAAAGTAGC-3′	591	58	28	26–30
			Reverse	5′-AAGTGGGCAAGGAGACAGAAAGTAAGTA-3′				
BDNF	*Bdnf*	NM_001270630	Forward	5′-AGGGGCATAGACAAAAGGCACTG-3′	403	59	23	21–25
			Reverse	5′-AACGGCAACAAACCACAACATTAT-3′				
GAPDH	*Gapdh*	NM_017008	Forward	5′-TATGACTCTACCCACGGCAAGTTCAA-3′	830	60	18	16–24
			Reverse	5′-ACCACCCTGTTGCTGTAGCCATATTCAT-3′				

Exponential Amplification: PCR cycle numbers for the exponential amplification phase, obtained by using the 3 m hippocampal cDNA templates

bp Base pair, T_a annealing temperature

between PCR products and target sequences was confirmed with DNA sequencing.

Comparison of the mRNA levels for different enzymes and receptors

We used the comparison method for mRNA levels of different enzymes/receptors obtained by using different primers [15]. Relative abundance of different genes was estimated by adopting the expression level of glyceraldehyde-3-phosphate dehydrogenase mRNA (*Gapdh*) as an internal standard. Optical density value in each band was divided by the number of $(1 + e)^c$, where c is a PCR cycle number and e is an amplification efficacy obtained from the PCR amplification curves in the exponential amplification phase. For more theoretical details, see supplementary materials.

Statistical analysis

Data are expressed as mean±SEM. The mRNA levels at different ages were compared by one-way ANOVA followed by Tukey's post hoc test when significant differences were found. A $p < 0.05$ was considered to be statistically significant.

Results

Age-induced change in expression levels of mRNAs encoding sex-steroidogenic enzymes and sex-steroid receptors

We performed the PCR analysis in three brain subregions, including the CC, Hy and CL for male rats, which were aged 3 months (3 m), 12 months (12 m), and 24 months (24 m). In the following the *italic* name (e.g., *Gapdh*) means the gene name of a protein (e.g., GAPDH). The expression levels of *Gapdh* mRNA did not show a significant age-related change over 3–24 m (Fig. 1). The *Gapdh* levels were not different among these three brain subregions, within experimental error. Therefore, the expression levels of the respective enzymes and receptors can be normalized by *Gapdh*. Furthermore, the level of each enzyme and receptor was normalized by enzymes/receptors levels in 3 m Hi, which were set to be 100 %.

Although the patterns of age-related changes of mRNAs are complex, we could categorize them into four types: (1) monotonous decrease-type (2) monotonous increase-type (3) no change-type, and (4) up and down-type.

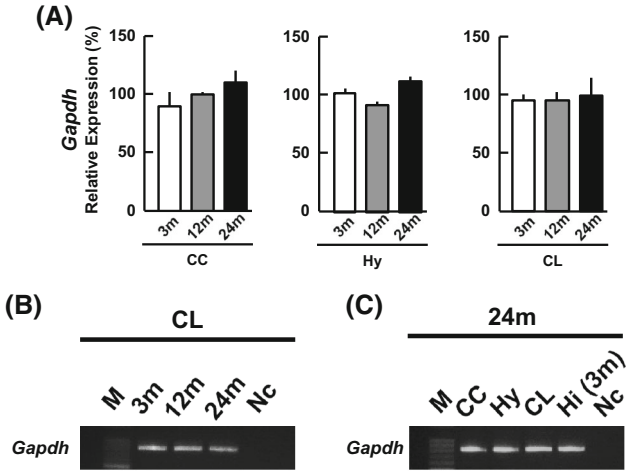


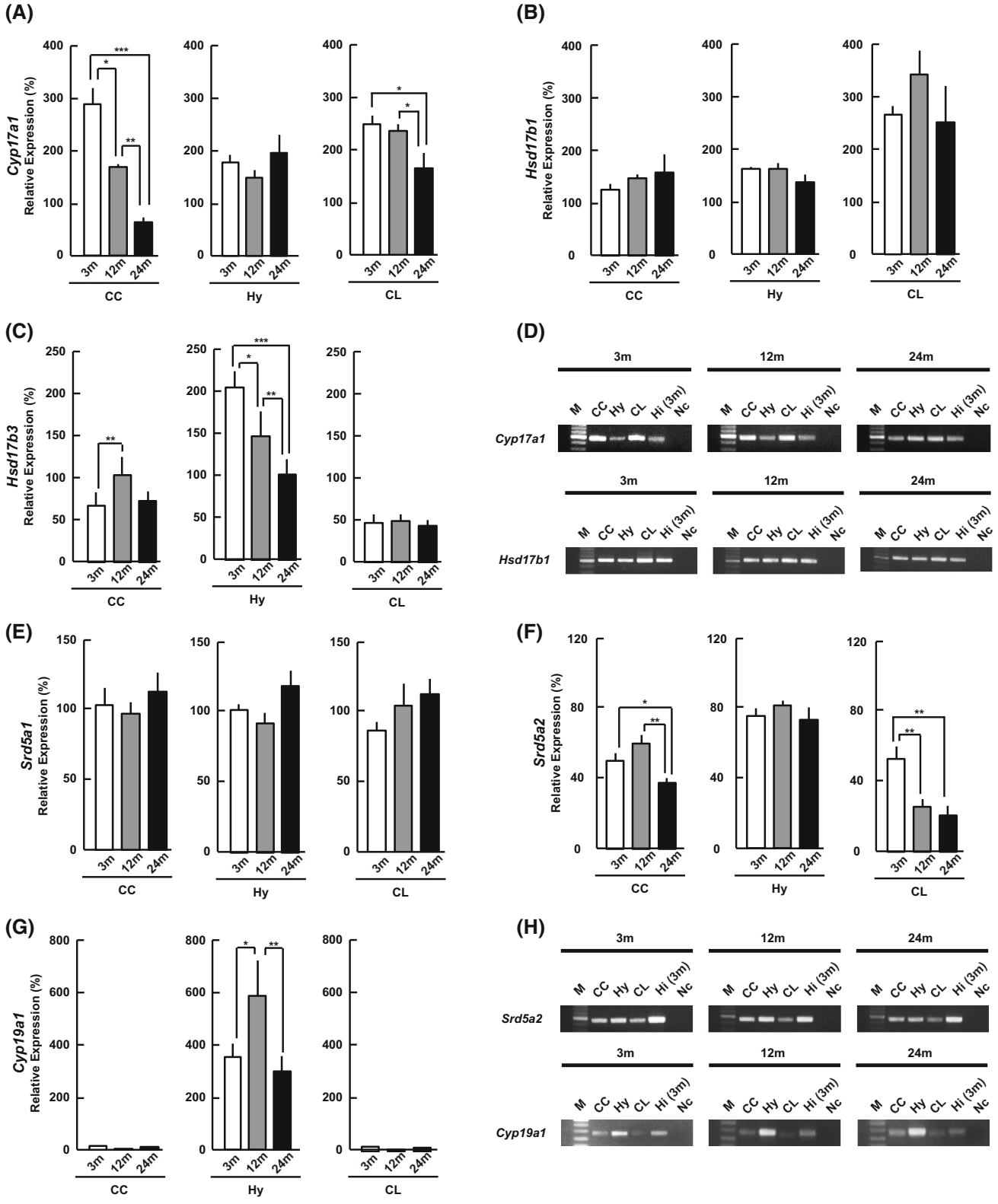
Fig. 1 mRNA expression of *Gapdh* (internal control) in the cerebral cortex (CC), hypothalamus (Hy), and cerebellum (CL) at 3, 12 and 24 m. **a** The *vertical axis* indicates the expression level for *Gapdh* calculated from the intensity of ethidium bromide bands. The expression level of each mRNA was normalized by 3 m hippocampus (Hi) which is set to be 100 %. Each value is mean ± SEM. Data are taken from duplicate determinations for each brain subregion of 4 rats. **b** A typical *Gapdh* PCR image for the CL. From *left* to *right*, 100-bp DNA ladder (*M*), 3, 12, 24 m, the sample without template cDNA as negative control (*Nc*). **c** A typical *Gapdh* PCR image at 24 m. From *left* to *right*, 100-bp DNA ladder (*M*), *CC, Hy, CL, 3 m Hi* (positive control), negative control (*Nc*). The PCR products are visualized with ethidium bromide

Fig. 2 mRNA expression of the steroidogenic enzymes [**a** *Cyp17a1,*▶ **b** *Hsd17b1,* **c** *Hsd17b3,* **e** *Srd5a1,* **f** *Srd5a2,* **g** *Cyp19a1*] at 3, 12, and 24 m. The *vertical axis* indicates the expression level for each enzyme calculated from the intensity of ethidium bromide bands. The expression level of each mRNA was normalized with *Gapdh* and the corresponding mRNA expression in 3 m *Hi*, as described in Methods. Each value is mean ± SEM. Statistical significance, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Data are taken from duplicate determinations for each brain subregion of 4 rats. **d**, **h** Typical PCR images for (**d**) *Cyp17a1* and *Hsd17b1*, and (**h**) *Srd5a2* and *Cyp19a1*. In each panel, from *left* to *right*, 100-bp DNA ladder (*M*), *CC, Hy, CL, 3 m Hi* (positive control), the sample without template cDNA (negative control, *Nc*)

Enzymes for androgen/estrogen synthesis

Cytochrome P450(17α) [Cyp17a1]

P450(17α) converts pregnenolone (PREG) to dehydroepiandrosterone (DHEA), or/and progesterone (PROG) to androstenedione (ADione). *Cyp17a1* expression level in the CC monotonously decreased with age, from 279 % (3 m) to 163 % (12 m), and 62 % (24 m) (Fig. 2a). In the Hy, the *Cyp17a1* level did not alter significantly with age. In the CL, the mRNA level at 24 m was lower than that at 3 m.


(A)
Cyp17a1
Relative Expression (%)
CC
Hy
CL
(B)
Hsd17b1
Relative Expression (%)
(C)
Hsd17b3
Relative Expression (%)
(D)
3m
12m
24m
M
CC
Hy
CL
Hi (3m)
Nc
Cyp17a1
Hsd17b1
(E)
Srd5a1
Relative Expression (%)
(F)
Srd5a2
Relative Expression (%)
(G)
Cyp19a1
Relative Expression (%)
(H)
Srd5a2
Cyp19a1

17β-hydroxysteroid dehydrogenase (17β-HSD) type 1 [Hsd17b1] and type 3 [Hsd17b3]

17β-HSDs (1 and 3) convert DHEA to androstenediol (ADiol), androstenedione (ADione) to T, or estrone E1 to E2. The *Hsd17b1* levels did not significantly change with age in the three subregions (Fig. 2b).

On the other hand, the expression level of *Hsd17b3* in the Hy monotonously decreased with age from 202 % (3 m) to 100 % (24 m) (Fig. 2c). The *Hsd17b3* level in the CC showed up-and-down change with age. In the CL, the *Hsd17b3* level did not show age-related change.

5α-reductase 1 [Srd5a1] and 5α-reductase 2 [Srd5a2]

5α-reductases (1 and 2) convert T to dihydrotestosterone (DHT). The *Srd5a1* mRNA expression levels did not significantly change with aging in all three subregions (Fig. 2e).

On the other hand, the *Srd5a2* expression level decreased with age, from 50 % (3 m) to 37 % (24 m). The *Srd5a2* level in the CL showed age-related monotonous decrease from 3 to 24 m as follows: 52 % (3 m), 26 % (12 m) and 21 % (24 m) (Fig. 2f). However, the *Srd5a2* level in the Hy did not show a significant change with age.

Cytochrome P450aromatase (P450arom) [Cyp19a1]

P450arom converts T to E2. Interestingl, in the CC and CL, the expression level of *Cyp19a1* was nearly negligible (below 5 %), over 3, 12 and 24 m, suggesting almost no estradiol synthesis (Fig. 2g). On the other hand, the Hy had a high *Cyp19a1* expression level and showed up-and-down change with aging as follows: 340 % (3 m), 563 % (12 m), and 288 % (24 m).

Comparison of enzymes between three subregions of 24 m brain

The expression level of *Cyp17a1* in the CC was lower than that in the Hy and CL (Fig. 2a). The expression level of *Hsd17b1* in the CL was approximately 2-fold that in the CC and Hy (Fig. 2b). *Srd5a1* was nearly the same in three brain subregions (Fig. 2e). The relative abundance of *Hsd17b3* (Fig. 2c) and *Srd5a2* (Fig. 2f) mRNAs were in the following order: Hy > CC ≥ CL. The *Cyp19a1* levels in the CC and CL were very low (Fig. 2g).

Receptors for androgen and estrogen

Androgen receptor (AR) [Ar]

Interestingly, in the Hy, the *Ar* level increased monotonously with age, from 120 % (3 m), to 145 % (12 m) and 151 % (24 m) (Fig. 3a). The level of *Ar* in the CC decreased from 3 to 24 m. The *Ar* level in the CL did not change with age.

Estrogen receptor ERα [Esr1] and ERβ [Esr2]

The *Esr1* expression level in the CC and Hy increased from 3 to 12 m and 24 m. In the CL, *Esr1* did not change with age (Fig. 3b).

The *Esr2* expression levels in the CC and Hy did not change with age, but *Esr2* in the CL monotonously decreased with age (Fig. 3c).

Comparison of sex-steroid receptors between three subregions of 24 m brain

The mRNA levels of *Ar* (Fig. 3a), *Esr1* (Fig. 3b) and *Esr2* (Fig. 3c) were higher in the Hy than in the CC and CL. The *Ar* level in the Hy was approximately 4-fold that in the CC and CL. The *Esr1* level in the Hy was approximately 10- to 15-fold that in the CC and CL.

Cholesterol transport proteins

Steroidogenic acute regulatory protein (StAR) and translocator protein (TSPO) transport cholesterol to the mitochondrial inner membrane from the cytoplasm.

StAR [Star]

The *Star* expression level did not change in the CC (Fig. 4a). The *Star* levels showed down-and-up change with age in the Hy and CL.

TSPO [Tspo]

The *Tspo* level did not show age-related change in the CC and Hy (Fig. 4b). On the other hand, the *Tspo* level showed down-and-up change in the CL.

Comparison of transport proteins between three subregions of 24 m brain

The expression level of *Star* was CL > Hy > CC (Fig. 4a). The *Tspo* expression level in the Hy was 2- to 3-fold higher than that in the CC and CL (Fig. 4b).

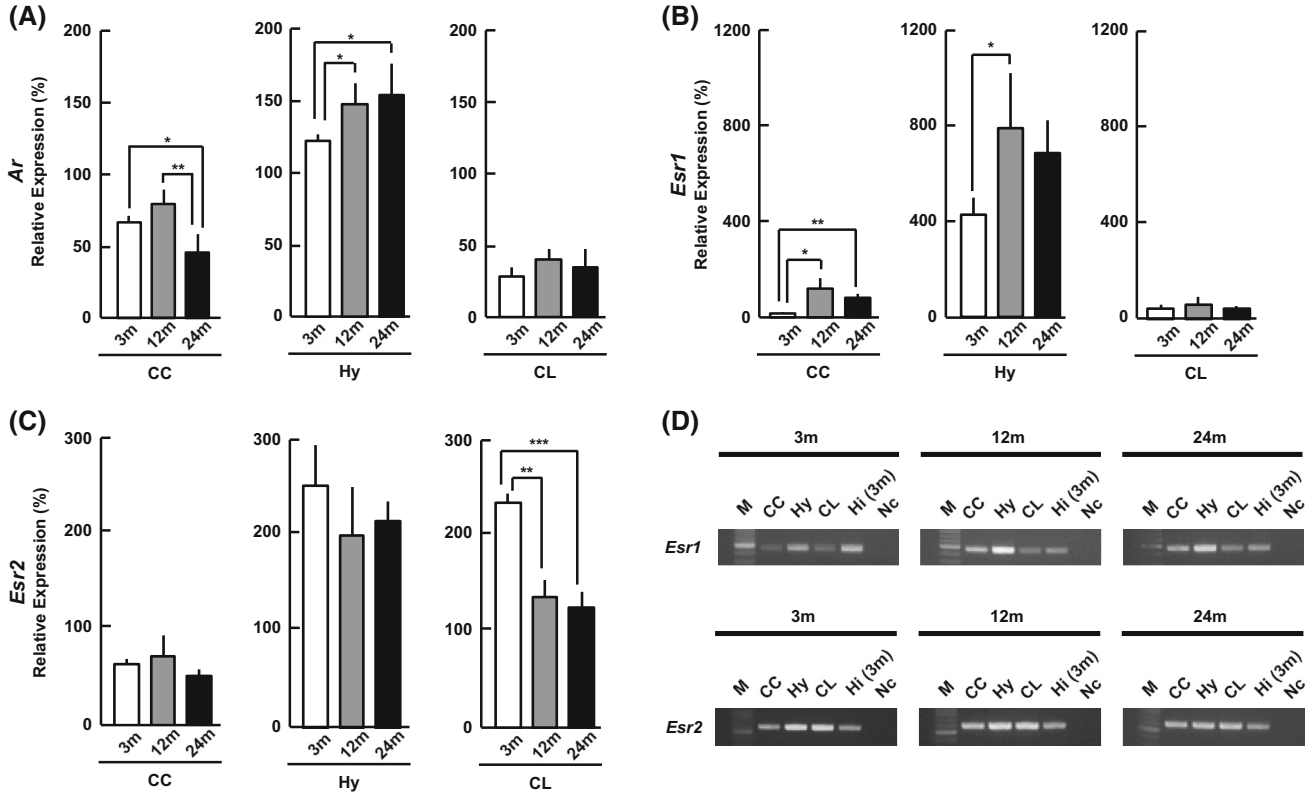


Fig. 3 mRNA expression of sex-steroid receptors [**a** *Ar,* **b** *Esr1,* **c** *Esr2*] at 3, 12, and 24 m. The *vertical axis* indicates the expression level for each receptor calculated from the intensity of ethidium bromide bands. Each value is mean±SEM. Statistical significance, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Data are taken from duplicate determinations for each brain subregion of 4 rats. **d** Typical PCR images for *Esr1* and *Esr2*. In each panel, from *left* to *right*, 100-bp DNA ladder (*M*), *CC, Hy, CL, 3 m Hi* (positive control), no template DNA (negative control, *Nc*). The normalization of each mRNA was performed in the same manner as Fig. 2

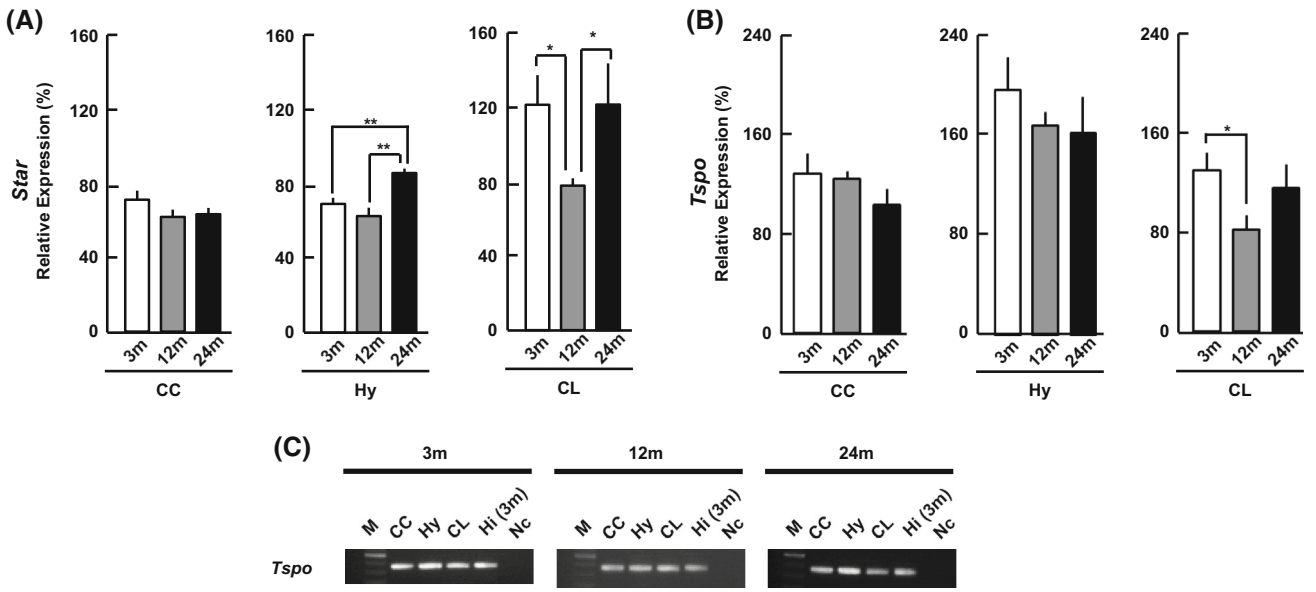


Fig. 4 mRNA expression of *Star* and *Tspo* in 3, 12, and 24 m. **a, b** The *vertical axis* indicates the expression level for each enzyme calculated from the intensity of ethidium bromide bands. Each value is mean±SEM. Statistical significance, $*p < 0.05$, $**p < 0.01$. Data are taken from duplicate determinations for each brain subregion of 4 rats. **c** Typical PCR images for *Tspo*. In each panel, from *left* to *right*, 100-bp DNA ladder (*M*), *CC*, *Hy*, *CL*, *Hi*, no template DNA (negative control, *Nc*). The normalization of each mRNA was performed in the same manner as Fig. 2

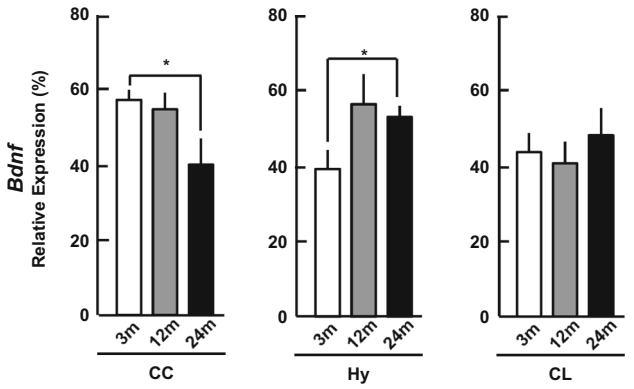


Fig. 5 mRNA expression of *Bdnf* in 3, 12 and 24 m. The *vertical axis* indicates the expression level calculated from the intensity of ethidium bromide bands. Each value is mean ± SEM. Statistical significance, $*p < 0.05$. Data are taken from duplicate determinations for each brain subregion of 4 rats. The normalization of each mRNA was performed in the same manner as Fig. 2

Brain-derived neurotrophic factor [*Bdnf*]

Rat *Bdnf* mRNAs contain at least nine splice variants. To detect all splice variants, we designed the Bdnf primers in the common region. The *Bdnf* expression level decreased from 3 to 24 m in the CC, but increased in the Hy and did not significantly change in the CL (Fig. 5). The relative abundance of *Bdnf* mRNAs in 24 m brain subregions was in the following order: Hy ∼ CL > CC (Fig. 5).

Comparison of all different enzymes and receptors in 24, 12, 3 m brains

We compared relative expression levels of all enzymes and receptors, examined in the current study (Fig. 6). Since *Cyp19a1* expression was the lowest among all the enzymes and receptors, we normalized by setting *Cyp19a1* in 3 m Hi

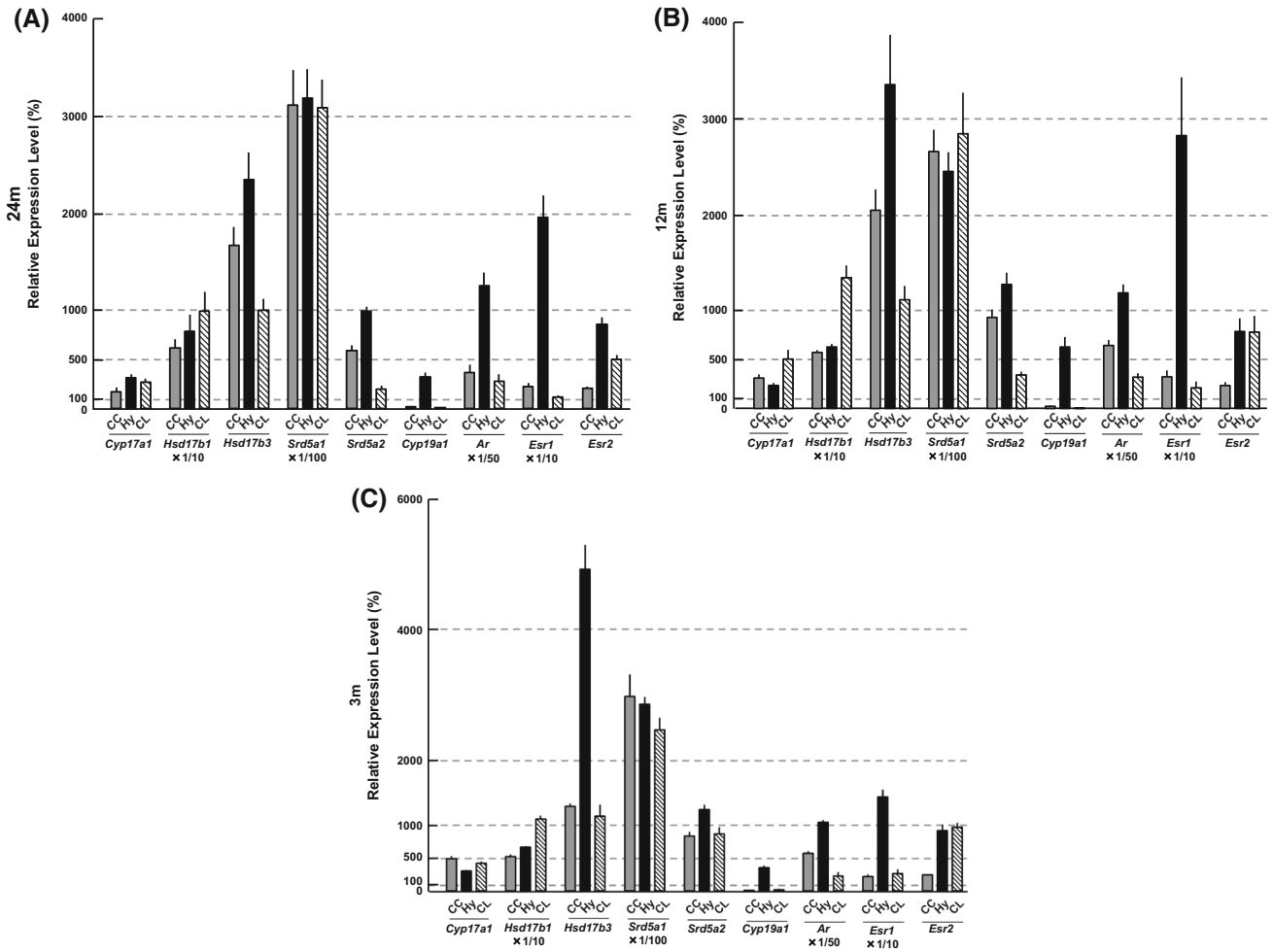


Fig. 6 Comparison of expression levels of mRNAs between different enzymes and receptors in (**a**) 24 m, (**b**) 12 m and (**c**) 3 m brains. The comparison was performed using the method as described in Materials and methods. The normalization of each mRNA was performed by setting *Cyp19a1* of 3 m *Hi* as 100 %. Data are also normalized by *Gapdh*. The *vertical scale* is different for each enzyme/receptor. For comparison, the *vertical scale* should amplify by 100-fold for *Srd5a1*; by 50-fold for *Ar*; by 10-fold for *Hsd17b1* and *Esr1*; by 1-fold for *Cyp17a1*, *Hsd17b3*, *Srd5a2*, *Cyp19a1* and *Esr2*. The data at 3 m was modified from Kimoto et al. [15]

to be 100 %. Hippocampal *Cyp19a1* was chosen as the standard, since this kind of comparison was already demonstrated for 3 m Hi elsewhere [15].

Several interesting characteristics can be seen for 24 m in Fig. 6. *Hsd17b1* was higher (by 3- to 10-fold) than *Hsd17b3*. *Srd5a1* was much higher (by 300- to 1,500-fold) than *Srd5a2*. *Esr1* was higher than *Esr2*, by ~10-fold in the CC, by ~20-fold in the Hy and by ~2-fold in the CL. In the CC and CL, enzymes for T synthesis were expressed at relatively high levels, but *Cyp19a1* for E2 synthesis was extremely low, suggesting almost no estradiol synthesis in the CC and CL. As a comparison, relative expression levels of all the enzymes and receptors in 12 and 3 m are also shown.

Discussion

Only a few investigations have been accumulated about age-related changes in sex-steroidogenic enzymes and sex-steroid receptors in the CC, Hy and CL, although many earlier investigations have been performed for age-induced changes in glutamate receptors and postsynaptic density protein 95 (PSD-95) in the CC, Hy, CL and the hippocampus [5, 24]. In addition, information for normal aging has not been sufficiently accumulated, compared with investigations for Alzheimer's disease.

In the current study, we demonstrated age-related changes of mRNA expressional levels of sex-steroidogenic enzymes/receptors in important brain subregions (CC, Hy, and CL). The age-induced changes of mRNAs are complex, but can be categorized into four patterns: (1) monotonous decrease, (2) monotonous increase, (3) no change, and (4) up and down change.

Androgen synthesis systems

In many cases, the expression levels of mRNA decreased with age for androgen synthesis enzyme systems, including *Cyp17a1* and *Srd5a2* in the CC and CL. On the other hand, no decrease with age was observed for *Hsd17b(1,3)* and *Srd5a1* in the CC and CL. The expression level of *Srd5a1* was the highest among all steroidogenic enzymes, and much higher than *Srd5a2*. The catalytic activity of *Srd5a2* is, however, much higher (~100-fold) than *Srd5a1*, as judged from its Km value for substrates T or PROG [20]. Taken together, these results suggest that androgen synthesis activity in aged CC and CL may moderately decrease.

In the CC, *Cyp17a1* decreased to approximately 35 % and *Srd5a2* decreased to approximately 70 % at 24 m, therefore, the capacity of DHT synthesis might decrease to roughly 25 % ($0.35 \times 0.7 = 0.25$) with age. In the CL, *Cyp17a1* decreased to approximately 65 % and *Srd5a2* decreased to approximately 40 % at 24 m, therefore, the capacity of DHT synthesis might decrease to roughly 25 % ($0.65 \times 0.4 = 0.26$) with age. In the Hy, only *Hsd17b3* level decreased to approximately 50 % at 24 m, but *Cyp17a1*, *Hsd17b1* and *Srd5a(1, 2)* did not show age-related change. Therefore, the capacity of DHT synthesis might decrease to roughly 50 % with age.

Only a few studies have been published on steroidogenic enzymes in the brains of aged animals [16, 29]. In the prefrontal cortex, Hy and Hi, mRNAs of *3BHSD1/2*, *17BHSD(1,3,5)* are expressed in middle-aged macaque monkeys (approximately 10 years old) [29]. The level of *17BHSD(1,3)* was, however, extremely low. When the CL and Hi of Alzheimer's disease patients and normal aging humans were compared, no significant differences were observed for *StAR*, *CYP11A1*, *HSD3B2*, *HSD17B(1,3,5)* and *SRD5A2* [16].

Only a few studies have been published for StAR expression [27]. In 24 m rat Hi, StAR protein immunoreactivity was higher than that in 2 m Hi. These results may not be different the current observation that *Star* mRNA level did not decrease with age in the CC, Hy and CL (Fig. 4a).

Estrogen synthesis enzyme P450arom

The capacity of E2 synthesis in the Hy may not considerably decrease with age, because *Cyp19a1* did not decrease, and only *Hsd17b3* decreased with age in the Hy. Interestingly, in the CC and CL, almost no expression of *Cyp19a1* was observed over 3–24 m, implying that the activity of estrogen synthesis in the CC and CL would be very weak. The CC and CL of adult and aged rats might need E2 supply from other regions, including the Hy or Hi, via diffusion/penetration of E2, since the Hi and Hy could have relatively high activity of E2 synthesis [8]. Penetration of low level E2 (~0.02 nM) from blood plasma may not be sufficient in the male brain. On the other hand, E2 may be sufficient in the CL in the neonatal stage, since *Cyp19a1* is significantly expressed in the CL at the neonatal stage [26].

Androgen and estrogen receptors

Interestingly, the expression level of *Ar* in the Hy monotonously increased with age (Fig. 3). This may be consistent with the previous study in which the number and density of AR-positive cells in the Hy increased during aging [31]. On the other hand, the *Ar* level in the CC decreased and that in the CL did not change (Fig. 3). These results may be somewhat different from earlier works that show no change of *Ar* mRNA level in rat CC and Hy [14].

Age-induced moderate increase in *Esr1* (ERα mRNA) in the Hy was observed (Fig. 3). Age-related change of ERα mRNA has been examined in subregions of the Hy [30]. *Esr1* levels do not change in the medial preoptic nucleus, arcuate nucleus, and ventromedial nucleus, but *Esr1* decreases in the periventricular preoptic nucleus [30].

The expression level of *Esr2* (ERβ mRNA) did not change in the CC and Hy with age, but decreased in the CL (Fig. 3). An earlier study also implies no change of *Esr2* in the CC and Hy, and decrease in the CL with age [32]. Age-related change of *Esr2* has been examined in subregions of the Hy [30]. *Esr2* does not change with age in the paraventricular nucleus, but *Esr2* decreases in the periventricular preoptic nucleus, medial preoptic nucleus, and paraventricular nucleus.

Although the expression level of *Esr1* was low in the CC and CL, compared with the Hy, moderate expression of *Esr2* in these regions may support sufficient function of (ERα+ERβ) in the aged state (Fig. 6).

Enzymes and receptors in the Hy often show no decline or increase with age

With aging of rat Hy, mRNAs of P450arom, 5α-reductase, and ERβ do not decrease [32], and AR and ERα increase, by going from 3 to 24 m. Since the sealing of the blood–brain barrier in the Hy is much looser than that in other brain regions, including the CC and CL, the decline in plasma sex steroids may induce resistance against the tendency of age-induced decrease in receptors and steroidogenic enzymes in the Hy. In the Hy, the number of ERα-immunoreactive cells and AR-immunoreactive cells increase with age [31]. These age-resistant phenomena might occur through the hypothalamus–pituitary–gonadal axis during the aging processes, due to strong interactions between plasma sex steroids and the Hy.

Neurotrophic factors

Neurotrophic factors as well as sex steroids play important roles in neuroprotection and maintenance of neural activity. In the Hy and CL, *Bdnf* did not decrease (Fig. 5), therefore age-dependent decrease in sex-steroidogenesis may play an important role in the impairment or deficiency of neural functions with age. The CC may be most sensitive to aging, since both sex-steroidogenic enzymes and *Bdnf* decreased with age (current study).

There have been many earlier reports concerning BDNF protein and mRNA. In rat CC, BDNF protein shows a decrease [12] or no change [23] with age. In the Hy and CL, BDNF protein and mRNA levels do not change with age, which is consistent with the current observation (Fig. 5) [3, 12, 23, 28].

Comparison between semi-quantitative PCR and real-time PCR

We can obtain essentially the same information from the current semi-quantitative PCR method [7, 9, 15] and real-time PCR [19]. In order to perform normalization, both methods need to choose a good standard housekeeping gene, which must not change with age in addition to region-independent expression. The real-time PCR also cannot avoid these processes. We observed that *Gapdh* satisfied these criteria. Since the expression level of mRNAs for steroidogenic enzymes is extremely low [7, 9, 15], the primer design is most important. The primer design (with specificity and selectivity) is dependent on the free energy calculation. Our semi-quantitative PCR methods have been better-optimized (from much experience) than real-time PCR (with less experience). Since we can choose the PCR cycle number within the exponential amplification phase, we can achieve essentially the same results with the semi-quantitative PCR method and the real-time PCR method.

References

1. Breslauer KJ, Frank R, Blocker H, Marky LA (1986) Predicting DNA duplex stability from the base sequence. Proc Natl Acad Sci USA 83:3746–3750
2. Chen H, Hardy MP, Huhtaniemi I, Zirkin BR (1994) Age-related decreased Leydig cell testosterone production in the brown Norway rat. J Androl 15:551–557
3. Ewa BS, Beata L, Ilona K, Dariusz S, Janusz M (2012) Brain derived neurotrophic factor (BDNF) containing neurons in the hypothalamic paraventricular and supraoptic nuclei of juvenile and middle-aged rats after chronic stress. Int J Dev Neurosci Off J Int Soc Develop Neurosci 30:139–146
4. Foy MR, Xu J, Xie X, Brinton RD, Thompson RF, Berger TW (1999) 17beta-estradiol enhances NMDA receptor-mediated EPSPs and long-term potentiation. J Neurophysiol 81:925–929
5. Gore AC, Oung T, Woller MJ (2002) Age-related changes in hypothalamic gonadotropin-releasing hormone and N-methyl-D-aspartate receptor gene expression, and their regulation by oestrogen, in the female rat. J Neuroendocrinol 14:300–309
6. Hao J, Rapp PR, Leffler AE, Leffler SR, Janssen WG, Lou W, McKay H, Roberts JA, Wearne SL, Hof PR, Morrison JH (2006) Estrogen alters spine number and morphology in prefrontal cortex of aged female rhesus monkeys. J Neurosci Off J Soc Neurosci 26:2571–2578
7. Hojo Y, Hattori T, Enami T, Furukawa A, Suzuki K, Ishii HT, Mukai H, Morrison JH, Janssen WGM, Kominami S, Harada N, Kimoto T, Kawato S (2004) Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017 alpha and P450 aromatase localized in neurons. Proc Natl Acad Sci USA 101:865–870
8. Hojo Y, Higo S, Ishii H, Ooishi Y, Mukai H, Murakami G, Kominami T, Kimoto T, Honma S, Poirier D, Kawato S (2009) Comparison between hippocampus-synthesized and circulation-derived sex steroids in the hippocampus. Endocrinology 150:5106–5112

9. Hojo Y, Murakami G, Mukai H, Higo S, Hatanaka Y, Ogiue-Ikeda M, Ishii H, Kimoto T, Kawato S (2008) Estrogen synthesis in the brain—role in synaptic plasticity and memory. Mol Cell Endocrinol 290:31–43
10. Janmaat S, Akwa Y, Doulazmi M, Bakouche J, Gautheron V, Liere P, Eychenne B, Pianos A, Luiten P, Groothuis T, Baulieu EE, Mariani J, Sherrard RM, Frederic F (2011) Age-related Purkinje cell death is steroid dependent: rORalpha haplo-insufficiency impairs plasma and cerebellar steroids and Purkinje cell survival. Age (Dordr) 33:565–578
11. Kato A, Hojo Y, Higo S, Komatsuzaki Y, Murakami G, Yoshino H, Uebayashi M, Kawato S (2013) Female hippocampal estrogens have a significant correlation with cyclic fluctuation of hippocampal spines. Front Neural Circuits 7:149
12. Katoh-Semba R, Semba R, Takeuchi IK, Kato K (1998) Age-related changes in levels of brain-derived neurotrophic factor in selected brain regions of rats, normal mice and senescence-accelerated mice: a comparison to those of nerve growth factor and neurotrophin-3. Neurosci Res 31:227–234
13. Kawato S, Hojo Y, Kimoto T (2002) Histological and metabolism analysis of P450 expression in the brain. Methods Enzymol 357:241–249
14. Kerr JE, Allore RJ, Beck SG, Handa RJ (1995) Distribution and hormonal regulation of androgen receptor (AR) and AR messenger ribonucleic acid in the rat hippocampus. Endocrinology 136:3213–3221
15. Kimoto T, Ishii H, Higo S, Hojo Y, Kawato S (2010) Semi-comprehensive analysis of the postnatal age-related changes in the mRNA expression of sex steroidogenic enzymes and sex steroid receptors in the male rat hippocampus. Endocrinology 151:5795–5806
16. MacKenzie SM, Dewar D, Stewart W, Fraser R, Connell JM, Davies E (2008) The transcription of steroidogenic genes in the human cerebellum and hippocampus: a comparative survey of normal and Alzheimer's tissue. J endocrinol 196:123–130
17. Midzak AS, Chen H, Papadopoulos V, Zirkin BR (2009) Leydig cell aging and the mechanisms of reduced testosterone synthesis. Mol Cell Endocrinol 299:23–31
18. Mukai H, Tsurugizawa T, Murakami G, Kominami S, Ishii H, Ogiue-Ikeda M, Takata N, Tanabe N, Furukawa A, Hojo Y, Ooishi Y, Morrison JH, Janssen WGM, Rose JA, Chambon P, Kato S, Izumi S, Yamazaki T, Kimoto T, Kawato S (2007) Rapid modulation of long-term depression and spinogenesis via synaptic estrogen receptors in hippocampal principal neurons. J Neurochem 100:950–967
19. Munetsuna E, Hojo Y, Hattori M, Ishii H, Kawato S, Ishida A, Kominami SAJ, Yamazaki T (2009) Retinoic acid stimulates 17 beta-estradiol and testosterone synthesis in rat hippocampal slice cultures. Endocrinology 150:4260–4269
20. Normington K, Russell DW (1992) Tissue distribution and kinetic characteristics of rat steroid 5 alpha-reductase isozymes. Evidence for distinct physiological functions. J Biol Chem 267:19548–19554
21. Ooishi Y, Mukai H, Hojo Y, Murakami G, Hasegawa Y, Shindo T, Morrison JH, Kimoto T, Kawato S (2012) Estradiol rapidly rescues synaptic transmission from corticosterone-induced suppression via synaptic/extranuclear steroid receptors in the hippocampus. Cereb Cortex 22:926–936
22. Ota H, Akishita M, Akiyoshi T, Kahyo T, Setou M, Ogawa S, Iijima K, Eto M, Ouchi Y (2012) Testosterone deficiency accelerates neuronal and vascular aging of SAMP8 mice: protective role of eNOS and SIRT1. PLoS One 7:e29598
23. Perovic M, Tesic V, Mladenovic Djordjevic A, Smiljanic K, Loncarevic-Vasiljkovic N, Ruzdijic S, Kanazir S (2013) BDNF transcripts, proBDNF and proNGF, in the cortex and hippocampus throughout the life span of the rat. Age (Dordr) 35:2057–2070
24. Pires RS, Real CC, Folador TS, Tellini NR, Torrao AS, Britto LR (2010) Differential response of AMPA and NMDA glutamate receptors of Purkinje cells to aging of the chicken cerebellum. Neurosci Lett 478:146–149
25. Rapp PR, Morrison JH, Roberts JA (2003) Cyclic estrogen replacement improves cognitive function in aged ovariectomized rhesus monkeys. J Neurosci Off J Soc Neurosci 23:5708–5714
26. Sakamoto H, Mezaki Y, Shikimi H, Ukena K, Tsutsui K (2003) Dendritic growth and spine formation in response to estrogen in the developing Purkinje cell. Endocrinology 144:4466–4477
27. Sierra A, Lavaque E, Perez-Martin M, Azcoitia I, Hales DB, Garcia-Segura LM (2003) Steroidogenic acute regulatory protein in the rat brain: cellular distribution, developmental regulation and overexpression after injury. Eur J Neurosci 18:1458–1467
28. Silhol M, Bonnichon V, Rage F, Tapia-Arancibia L (2005) Age-related changes in brain-derived neurotrophic factor and tyrosine kinase receptor isoforms in the hippocampus and hypothalamus in male rats. Neuroscience 132:613–624
29. Sorwell KG, Kohama SG, Urbanski HF (2012) Perimenopausal regulation of steroidogenesis in the nonhuman primate. Neurobiol Aging 33(1487):e1413–e1481
30. Wilson ME, Rosewell KL, Kashon ML, Shughrue PJ, Merchenthaler I, Wise PM (2002) Age differentially influences estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) gene expression in specific regions of the rat brain. Mech Ageing Dev 123:593–601
31. Wu D, Lin G, Gore AC (2009) Age-related changes in hypothalamic androgen receptor and estrogen receptor alpha in male rats. J Comp Neurol 512:688–701
32. Yamaguchi-Shima N, Yuri K (2007) Age-related changes in the expression of ER-beta mRNA in the female rat brain. Brain Res 1155:34–41