Biochemistry and Biophysics Reports 35 (2023) 101548

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Effect of estradiol replacement on hippocampal concentrations of estrogens in aged rhesus macaques maintained on an obesogenic diet

Laszlo Prokai [a], Vien Nguyen [a], Henryk F. Urbanski [b,c,*]

[a] Department of Pharmacology & Neuroscience, University of North Texas Health Science Center at Fort Worth, TX, 76063, USA
[b] Division of Neuroscience, Oregon National Primate Research Center, Beaverton, OR, 97006, USA
[c] Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR, 97239, USA

ARTICLE INFO

Keywords:
LC-MS/MS
Estrone
Estradiol
Rhesus macaque
Western-style diet

ABSTRACT

Replacement involving estrogens has proven efficacy at treating a wide range of disorders that develop with menopause or after surgical removal of the ovaries. Here, we tested whether an estradiol (E2) replacement paradigm that recapitulates physiological E2 levels in the circulation also recapitulates physiological E2 levels within the hippocampus. E2 was delivered continuously to old ovariectomized (OVX) rhesus macaques, maintained on a high-fat, high-sugar Western-style diet (WSD) for ~30 months, via subcutaneous implants; this resulted in physiological concentrations of both estrone (E1) and E2 in the circulation (determined by LC-MS/MS). Surprisingly, however, hippocampal concentrations of E2 were markedly ($P < 0.01$) higher than in ovary-intact animals maintained on a regular chow diet. The data suggest that E2 replacement paradigms that appear to recapitulate physiological E2 concentrations in the circulation may produce hyper-physiological E2 levels within some brain areas, especially when individuals are maintained on a WSD.

1. Introduction

Menopause is associated with a marked decrease in circulating concentrations of estrogens, which in turn contributes to the etiology of several age-associated disorders, including hot flushes [1,2], decreased bone mineral density [3,4], cognitive decline [5–9], increased risk of cardiovascular disease [10,11] and attenuated immune function [12,13]. Consequently, estrogen + progestin hormone replacement therapy (HRT) in postmenopausal women, and estrogen replacement therapy (ERT) in women that have had their ovaries surgically removed represent effective interventions [14,15]. Although HRT and ERT aspire to restore circulating concentrations of estrogens, such as estrone (E1) and estradiol (E2), to physiological concentrations observed in adult premenopausal women, it is unclear if estrogen concentrations within the brain are also restored to physiological levels, especially in women who regularly consume a high-fat, high-sugar, Western-style diet (WSD).

To address this issue, we used liquid chromatography–tandem mass spectrometry (LC–MS/MS) to measure E1 and E2 concentrations in serum and brain of old ovariectomized "surgically menopausal" rhesus macaques that had been maintained on a WSD for ~30 months, either with or without ERT. Like women, adult female rhesus macaques show menstrual cycles and show many similar age-related hormone changes, including a marked decrease in circulating concentrations of estradiol, progesterone and dehydroepiandrosterone sulfate [16–18]. Importantly, these long-lived primates can be maintained under tightly controlled environmental conditions, such as photoperiod, ambient temperature and diet [19]. Moreover, at necropsy, brain tissue can be collected with zero postmortem interval and can be immediately frozen for subsequent hormone measurement. The results raise a concern that ERT that produces physiological concentrations of estrogens in the circulation of postmenopausal women might be producing hyper-physiological concentrations of estrogens within some tissues, including the brain. In particular, the hippocampus is rich in estrogen receptors [20,21] and plays an important role in cognitive function [8,9]. Furthermore, the hippocampus has been shown to respond robustly to estrogen in female monkeys [20]. Therefore, because of its translational context to ERT, our aim in the present study was to focus on this brain area.

2. Methods

2.1. Animals

The study utilized blood and brain tissue from 32 aged female rhesus

* Corresponding author. Division of Neuroscience, Oregon National Primate Research Center, 505 NW 185th Avenue, Beaverton, OR, 97006, USA.
E-mail address: urbanski@ohsu.edu (H.F. Urbanski).

https://doi.org/10.1016/j.bbrep.2023.101548
Received 18 July 2023; Received in revised form 30 August 2023; Accepted 12 September 2023

macaques (*Macaca mulatta*) that were part of several unrelated Institutional Animal Care and Use Committee approved research projects. The animals had been maintained on photoperiods comprising 12 h light and 12 h of darkness per day, and cared for in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*. Euthanasia was performed following an established protocol recommended by the American Veterinary Medical Association *Guidelines for the Euthanasia of Animals*, and postmortem tissues, were subsequently obtained through the ONPRC Tissue Distribution Program.

The animals were assigned to one of four experimental groups (Table 1). Eleven of the animals served as ovary-intact controls, while the others were ovariectomized (OVX) and either remained on a regular monkey chow diet ($n = 7$) (Monkey Diet, LabDiet, Inc., St Louis, MO, USA) or were exposed for ~30 weeks to a high-fat-high-sugar Western-style diet (WSD) (TAD Primate Diet; LabDiet, Inc.), which provided calories with 36% fat, 44% carbohydrates (includes 18.5% sugars) and 18% protein. In comparison, regular monkey chow provides calories with 13% fat, 69% complex carbohydrates (includes 6% sugars) and 18% protein; half of the OVX WSD animals ($n = 7$) received ERT by E2 in the form of subcutaneous elastomer capsules, beginning immediately after ovariectomy, while the other half ($n = 7$) received empty subcutaneous capsules [22,23]. The E2 capsules were designed to last up to 1 year and to maintain serum E2 concentrations between 100 and 200 pg/mL, which is similar to concentrations during the mid-to-late follicular phase of the menstrual cycle [16,24].

2.2. Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

Brain tissue (40–50 mg wet weight) were homogenized in 400 μL pH 7.4 phosphate buffer saline solution, diluted with 400 μL HPLC-grade water, spiked with 100 pg of $^{13}C_6$-E2 and 100 pg of $^{13}C_6$-E1 as internal standards, and extracted with 1.5 mL *tert*-butyl methyl ether. After centrifugation at 2500 rpm for 10 min, the organic layers were removed and transferred into reacti-vials (7 mL, Supelco, Bellefonte, PA, USA) for evaporation under a nitrogen stream. The residues were derivatized through the addition of 30 μL of 1 mg/mL dansyl chloride solution in acetonitrile and 20 μL of aqueous sodium bicarbonate (100 mM, pH 10.5) solution [25]. After incubation at 60 °C for 20 min, the dansylated samples were centrifuged at 13400 rpm for 5 min, transferred to autosampler vials, sealed, and assayed by LC–MS/MS. Sera (250 μL aliquots) were extracted and prepared for analyses similarly.

Estrogen (E2 and E1) quantitations were done by an LC–MS/MS assay based on the principles of isotope dilution [22,25,26]. Gradient LC separations were carried out on a Kinetex phenyl-hexyl column (100 Å, 50 × 2.1 mm, 1.7 μm) from Phenomenex (Torrance, CA, USA) operated at 0.4 mL/min flow rate using a Vanquish ultrahigh performance liquid chromatography (UHPLC) system (Thermo Electron Corporation, Trace Chemical Analysis, Austin, TX, USA) [26]. The eluent was from (A) 0.1% (*v/v*) formic acid in water and (B) 0.1% (*v/v*) formic acid in acetonitrile, as reported previously [25]. The mass spectrometer (TSQ Quantum Ultra, Thermo Electron Corporation) was operated in positive ion mode with a heated electrospray ionization (HESI) probe and using Xcalibur (version 4.0, Thermo Fisher Scientific, Waltham, MA, USA) data acquisition software. Selected reaction monitoring (SRM) with unit mass resolution for the precursor and product ions was used for quantification. SRM transitions of *m/z* 504 → 171, 506 → 171, 510 → 171 and 512 → 171 were set up for dansyl-E1, dansyl-E2, dansyl-$^{13}C_6$-E1 and dansyl-$^{13}C_6$-E2, respectively. Estrogen concentrations were expressed as pg/mL in serum and pg/g wet weight in brain tissue.

Table 1
Effect of Western-style diet and estrogens on body weight.

	n	Age (years)	Body weight (kg)
Ovary-intact	11	19.7 ± 1.8	7.1 ± 0.2
Ovariectomized	7	24.6 ± 1.2	7.8 ± 0.4
Ovariectomized (WSD)	7	22.4 ± 0.3	9.6 ± 0.4**#
Ovariectomized (WSD + ERT)	7	21.2 ± 0.5	8.7 ± 0.8*

Values represent means ± S.E.M. and n = number of animals per group. Mean ages were similar across the treatment groups (Kruskal-Wallis test) but some differences in body weight were detected by (ANOVA, ($F_{3,28} = 6.4584$, $P = 0.018$) followed by Newman-Keuls test).
*$P < 0.05$, **$P < 0.01$ (relative to ovary-intact group), #$P < 0.05$ (relative to ovariectomized group). ERT, 17β-estradiol (E2) hormone replacement therapy; WSD, Western-style diet.

2.3. Statistics

Each dataset was subjected to a Shapiro-Wilk test of normality. All parameters, except for animal ages, passed the test and so group means were subsequently analyzed using ANOVA followed by the Newman-Keuls test, while animal ages were compared using the Kruskal-Wallis non-parametric test. A P value of <0.05 was considered to be statistically significant.

3. Results

Animals in the four treatment groups had similar mean ages but, as expected, animals maintained on a WSD had higher mean body weights ($F_{3,28} = 6.4584$, $P = 0.018$; Table 1). Based on previous findings [22], serum concentrations of estrone (E1) and estradiol (E2) were typical of those observed during the early follicular phase of the menstrual cycle, as was the significant decrease in serum estradiol after ovariectomy [16,27]. Specifically, serum E2 levels showed significant differences between the treatment groups ($F_{3,28} = 26.6605$, $P < 0.0001$), with E2 levels being significantly ($P < 0.01$) lower in the two ovariectomized (OVX) groups than in the ovary-intact group or in the E2-treated animals (Fig.1A). However, serum E2 levels were significantly ($P < 0.01$) higher in the E2-treated animals than in the intact controls and were more similar to those levels previously observed in younger ovary-intact animals, especially during the mid-follicular to late-follicular phase of the menstrual cycle. Serum E1 levels also showed significant differences between the treatment groups ($F_{3,28} = 6.3601$, $P = 0.002$), with serum E1 levels being significantly ($P < 0.01$) lower in the two ovariectomized (OVX) groups than in the ovary-intact group, but not in the E2-treated group (Fig.1B), suggesting that the E2 treatment restored serum E1 to an ovary-intact physiological level. Hippocampal E2 levels showed significant differences between the treatment groups ($F_{3,28} = 47.1331$, $P < 0.0001$). The E2 levels were similar in the ovary-intact and OVX animals that had been maintained on a regular diet, as well as in the OVX (WSD) controls, but were significantly higher ($P < 0.01$) in the OVX (WSD) E2-treated animals and 11-fold higher than in the ovary-intact animals (Fig.1C). Although hippocampal E1 levels appeared to follow a similar trend to that of hippocampal E2 ($F_{3,28} = 3.2886$, $P = 0.035$), no significant between-treatment group differences were detected (Fig.1D).

4. Discussion

In adult women and female rhesus macaques, the ovary is the primary site of estrogen production, although there is evidence that some brain areas, such as the hippocampus, produce enzymes that can convert DHEA to estrogens [17]. It is plausible, therefore, that *de novo* estrogen synthesis in the brain helps to maintain physiological levels of estrogens in critical brain areas despite a marked decrease in circulating estrogen levels following menopause [28].

In the present study, we corroborated previous findings that ovariectomy of rhesus macaques results in a significant decrease in circulating concentrations of E1 and E2 [22]. In addition, we showed that a well-established estrogen replacement paradigm [22–24] restores estrogen concentrations to levels typically observed in the menstrual cycle [16]. Although the serum concentrations of E2 were significantly higher than those observed in the ovary-intact group, it needs to be emphasized that the levels were still well within the physiological ovary-intact

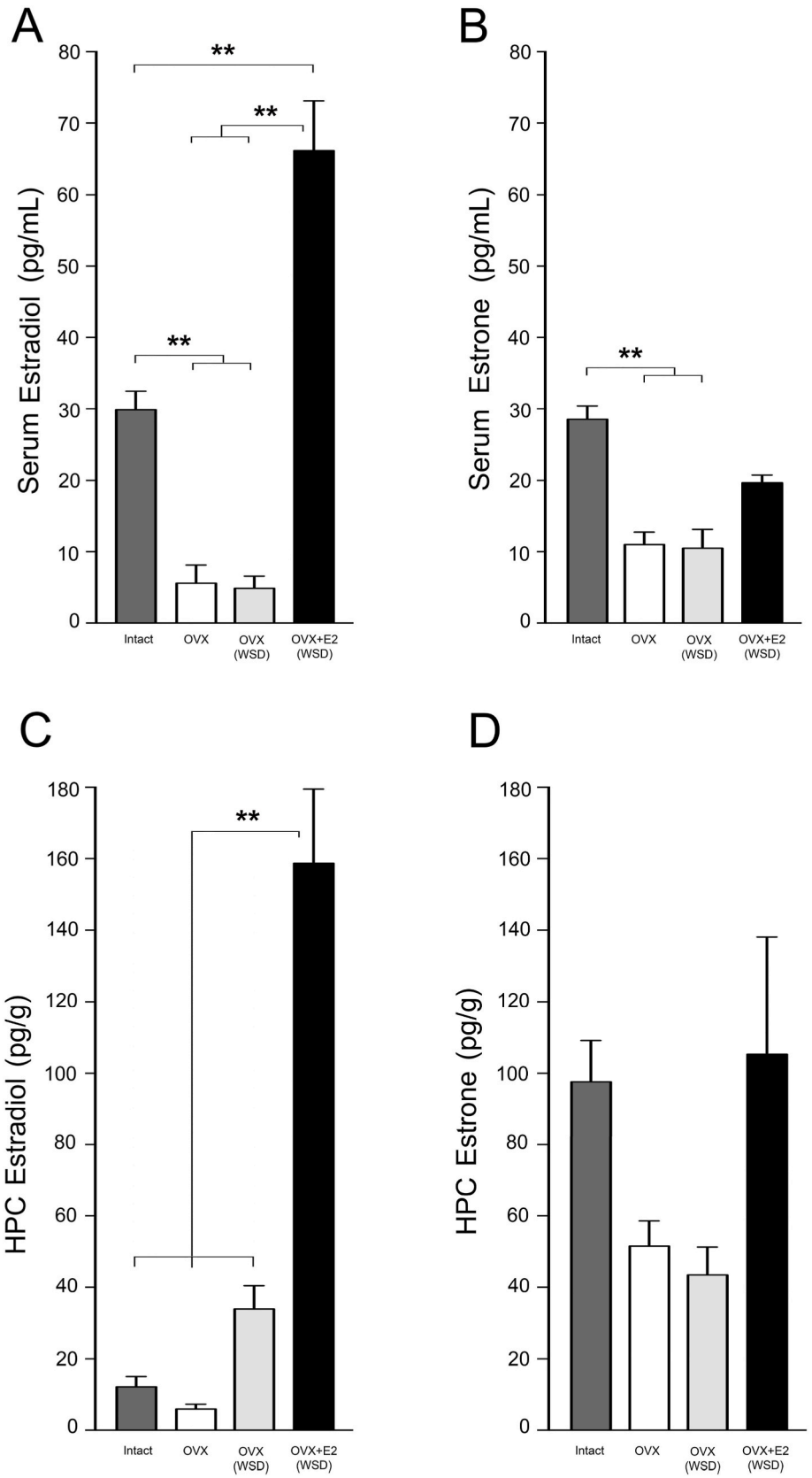

Fig. 1. Effect of ovariectomy and ERT on serum and HPC estrogen concentrations. Group mean values are represented by bars and SEMs are depicted as vertical lines. $^{**}P < 0.01$ (ANOVA followed by the Newman-Keuls test). OVX, ovariectomy; WSD, Western-style diet; E2, 17β-estradiol.

range, and more closely corresponding to levels typically observed in the mid- to late-follicular phase of the menstrual cycle, rather than to the early follicular or luteal phases [16]. More importantly, we showed for the first time that ERT results in hyper-elevated concentrations of E2, but not E1, in the hippocampus, despite maintaining serum E1 and E2 concentrations at a physiological level. It is unclear if similar hyper-elevation of E2 levels would occur in animals maintained on a regular diet or if the hyper-elevation is exacerbated in some way by the increased lipid environment of animals maintained on a WSD. It is also unclear if similar hyper-elevation of E2 occurs throughout the brain, as well as other organ systems, or only in the hippocampus. Nevertheless, because of similarities in brain structure and hormonal profiles of ovariectomized aged rhesus macaques and postmenopausal women, it is plausible that different clinically approved HRT regimens, may also produce hyper-physiological estrogen levels within the brain, despite maintaining circulating estrogen at a physiological level. It remains to be determined, however, whether such hyper-elevated brain estrogen concentrations are ultimately detrimental to health; they may actually be beneficial. Until postmortem clinical studies can be performed to evaluate these potential concerns, caution may be needed when prescribing HRT for postmenopausal symptoms and ERT after surgical menopause, especially to women on a WSD.

Verification

Assurance is given that this article is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by National Institutes of Health Grants: P30 AG066518, P51 OD011092, R01 AG070072, R24 OD011895 and RF1 AG062220, and by The Welch Foundation's endowment BK-0031.

References

[1] D.W. Sturdee, M.S. Hunter, P.M. Maki, P. Gupta, J. Sassarini, J.C. Stevenson, M. A. Lumsden, The menopausal hot flush: a review, Climacteric 20 (2017) 296–305.
[2] T.H. Mallhi, Y.H. Khan, A.H. Khan, Q. Mahmood, S.H. Khalid, M. Saleem, Managing hot flushes in menopausal women: a review, J. Coll. Physicians Surg. Pak. 28 (2018) 460–465.
[3] P.R. Ebeling, L.M. Atley, J.R. Gutherie, H.G. Burger, L. Dennerstein, J.L. Hopper, J. D. Wark, Bone turnover markers and bone density across the menopausal transition, J. Clin. Endocrinol. Metab. 81 (1996) 3366–3371.
[4] V. Seifert-Klauss, J.E. Mueller, P. Luppa, R. Probst, J. Wliker, C. Höss, T. Treumann, C. Kastner, K. Ulm, Bone metabolism during the perimenopausal transition: a prospective study, Maturitas 41 (2002) 23–33.
[5] A. Paganini-Hill, V.W. Henderson, Estrogen deficiency and risk of Alzheimer's disease in women, Am. J. Epidemiol. 140 (1994) 256–261.
[6] U. Halbreich, L.A. Lumely, S. Palter, C. Manning, F. Gengo, S.H. Joe, Possible acceleration of age effects on cognition following menopause, J. Psychiatr. Res. 29 (1995) 153–163.
[7] A. Lacreuse, Effects of ovarian hormones on cognitive function in nonhuman primates, Neuroscience 138 (2006) 859–867.
[8] P.M. Maki, E. Sundermann, Hormone therapy and cognitive function, Hum. Reprod. Update 15 (2009) 667–681.
[9] B.B. Sherwin, Estrogen and cognitive functioning in women: lessons we have learned, Behav. Neurosci. 126 (2012) 123–127.
[10] K.A. Do, A. Green, J.R. Guthrie, E.C. Dudley, H.G. Burger, L. Dennerstein, Longitudinal study of risk factors for coronary heart disease across the menopausal transition, Am. J. Epidemiol. 151 (2000) 584–593.
[11] K.A. Matthews, L.H. Kuller, K. Sutton-Tyrrell, Y.F. Chang, Changes in cardiovascular risk factors during the perimenopause and postmenopause and carotid artery atherosclerosis in healthy women, Stroke 32 (2001) 1104–1111.
[12] V.R. Porter, G.A. Greendale, M. Schocken, X. Zhu, R.B. Effros, Immune effects of hormone replacement therapy in post-menopausal women, Exp. Gerontol. 36 (2001) 311–326.
[13] J. Pfeilschifter, R. Köditz, M. Pfohl, H. Schatz, Changes in proinflammatory cytokine activity after menopause, Endocr. Rev. 23 (2002) 90–119.
[14] E. Armeni, S.A. Paschou, D.G. Goulis, I. Lambrinoudaki, Hormone therapy regimens for managing the menopause and premature ovarian insufficiency, Best Pract. Res. Clin. Endocrinol. Metabol. 35 (2021), 101561.
[15] R.D. Langer, H.N. Hodis, R.A. Lobo, M.A. Allison, Hormone replacement therapy - where are we now? Climacteric 24 (2021) 3–10.
[16] J.L. Downs, H.F. Urbanski, Neuroendocrine changes in the aging reproductive axis of female rhesus macaques (Macacaca mulatta), Biol. Reprod. 75 (2006) 539e546.
[17] K.G. Sorwell, S.G. Kohama, H.F. Urbanski, Perimenopausal regulation of steroidogenesis in the nonhuman primate, Neurobiol. Aging 33 (1487) (2012) e1–e13.
[18] K.G. Sorwell, H.F. Urbanski, Causes and consequences of age-related steroid hormone changes: insights gained from nonhuman primates, J. Neuroendocrinol. 25 (2013) 1062–1069.
[19] I. Messaoudi, H.F. Urbanski, S.G. Kohama, Integrative models of aging in the nonhuman primate, in: R.M. Williams (Ed.), Monkeys: Biology, Behavior and Disorders, Nova Science Publishers, Hauppauge, NY, 2011, pp. 1–54. Ch. 1.
[20] T.C. Register, C.A. Shively, C.E. Lewis, Expression of estrogen receptor α and β transcripts in female monkey hippocampus and hypothalamus, Brain Res. 788 (1998) 320–322.
[21] C. Gundlah, S.G. Kohama, S.J. Mirkes, V.T. Garyfallou, H.F. Urbanski, C.L. Bethea, Distribution of estrogen receptor beta (ERβ) mRNA in hypothalamus, midbrain and temporal lobe of spayed macaque: continued expression with hormone replacement, Mol. Brain Res. 76 (2000) 191–204.
[22] H.F. Urbanski, K.G. Sorwell, L. Prokai, S.G. Kohama, Effect of short-term DHEA supplementation on serum and hippocampal estrogen concentrations in perimenopausal female rhesus macaques, Neurobiol. Aging 55 (2017) 172–174.
[23] J.Q. Purnell, H.F. Urbanski, P. Kievit, C.T. Roberts, C.L. Bethea, Estradiol replacement timing and obesogenic diet effects on body composition and metabolism in postmenopausal macaques, Endocrinology 160 (2019) 899–914.
[24] C.L. Bethea, S.G. Kohama, A.P. Reddy, H.F. Urbanski, Ovarian steroids regulate gene expression in the dorsal raphe of old female macaques, Neurobiol. Aging 37 (2016) 179–191.
[25] S. Szarka, V. Nguyen, L. Prokai, K. Prokai-Tatrai, Separation of dansylated 17β-estradiol, 17α-estradiol, and estrone on a single HPLC column for simultaneous quantitation by LC–MS/MS, Anal. Bioanal. Chem. 405 (2013) 3399–3406.
[26] K. Prokai-Tatrai, V. Nguyen, D.L. De La Cruz, R. Guerra, K. Zaman, F. Rahlouni, L. Prokai, Retina-targeted delivery of 17β-estradiol by the topically applied DHED prodrug, Pharmaceutics 12 (2020) 456.
[27] D.H. Eghlidi, G.E. Haley, N.C. Noriega, S.G. Kohama, H.F. Urbanski, Influence of age and 17ß-estradiol on kisspeptin, neurokinin B, and prodynorphin gene expression in the arcuate-median eminence of female rhesus macaques, Endocrinology 151 (2010) 3783–3794.
[28] K.G. Sorwell, H.F. Urbanski, Dehydroepiandrosterone and age-related cognitive decline, Age (Dordr) 32 (2010) 61–67.