が海馬のERα発現量変化に起因している可能性も示した。また、その海馬の変化は幼若期から表れていることが示された。

生後24時間のEE曝露により、ERα発現量は、海馬ではエストロゲン存在下で、大脳皮質ではエストロゲン非存在下で減少した。一方ERβ発現量は、海馬ではエストロゲン非存在下で減少し、大脳皮質ではいずれの条件でも変化が認められなかった。さらに幼若期で認められた変化は必ずしも成熟期の変化と同様なものではなかった。従って、ER発現への生後24時間以内のEDs曝露の影響は脳領域、時期およびサブタイプにより異なり、さらにエストロゲンの機能的作用も部位特異的に変化させる可能性が示された。

生後28日間EEを経口投与した成熟ラットの床敷に対する接近行動を解析し、雌雄どちらの床敷に対して性選好性を示すかを調査した結果、雌ラットへのEE曝露は、雄床敷よりも雌床敷へ強い性選好性を示す、すなわち雄型の性選好性を示すことを明らかにした。成熟雄ラットとの性行動試験では、雌特異的な性行動である雄受容姿勢、誘惑行動に着目し解析した結果、雌ラットへのEE曝露により雄受容姿勢、誘惑行動のいずれも示さなくなることが明らかになった。このEE曝露濃度の一日当たりの投与量は、前プロジェクトでは性選好性試験や性行動試験に影響を及ぼさなかった生後24時間以内の20 μg/kg皮下投与より少なく、体内吸収も少ない経口投与であるが、反復して曝露することにより脳を雄型化する可能性が示唆された。

一方、生後28日間のTPhP経口投与は、雄ラットの性選好性スコアとの差を消失させることから、正常な雌型性選好性の成立を阻害する可能性が示唆された。雄との性行動試験においては、TPhP曝露は雄受容姿勢の発現に影響はないものの、高濃度のTPhP曝露では誘惑行動のうちHoppingの発現が減少する、すなわち曝露濃度により異なる影響があることが明らかになった。

成熟動物の一部の行動において抑制影響に性差が見られるジアゼパムを投与することにより、母子分離誘発啼鳴反応の性差を検出できるのではないかと考え検討し、雄子ラットのほうが雌仔ラットに比べ、ジアゼパムが引き起こす影響が強く現れることを明らかとした。このことから、母子分離誘発蹄鳴反応がEDsの早期指標となり得ることを示した。

E．結論

受動回避学習試験において、EEの遅発影響誘発量曝露による学習能力の低下がエストロゲン存在下で表れ、作用機序として海馬のERα発現量と関連する可能性が示された。また、ER発現へのエストロゲン様物質曝露の影響は、部位、時期およびサブタイプ特異的であることが改めて示された。

生後28日間のEE連続経口投与は、生後24時間以内の皮下投与と比べると低い濃度でも脳の正常な性分化に影響を与え、成熟後の雌特異的な性行動を抑制する可能性が示された。またTPhPは、曝露濃度依存的に特定の雌特異的な行動の発現を抑制する可能性が見出された。

加えて、母子分離誘発蹄鳴反応がEDsの早期指標となり得ることを示した。

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G．知的財産権の出願・登録状況（予定を含む。）

1. 特許取得
   該当なし
2. 実用新案登録
   該当なし
3. その他
   該当なし

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図1　Estrogen（E2）が引き起こす作用

1923-1928.

図2　E2が作用する中枢神経系の機能

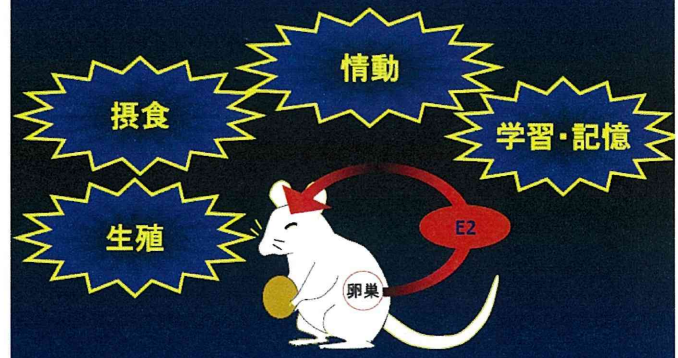


図3　縄張り行動試験

- 格子越しに相手ラットに接近できる3チャンバーケージを用いた。
- テストラット、雌雄相手ラットを、各部屋へ入れ、5分間観察した。

格子前にろ紙を敷き、尿を吸収させた。
ろ紙にニンヒドリン5 %試薬を散布し、染色された面積をImage Jを用いて解析した。

図4　受動回避学習試験装置

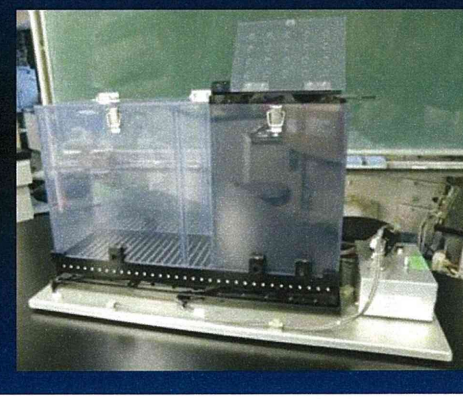


図5　母子分離誘発啼鳴反応試験

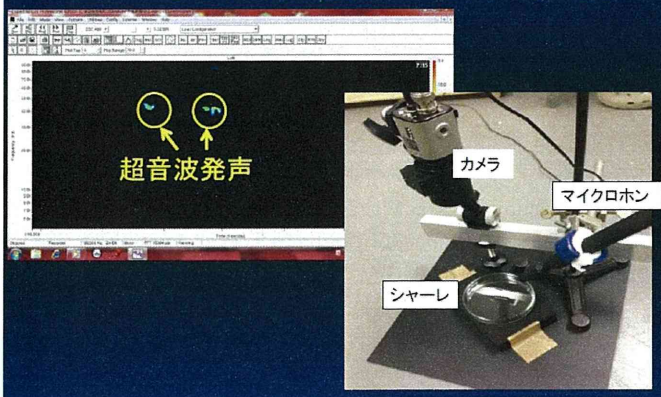


図6　縄張り行動試験結果

（Oil群における雄に対する尿面積＝1）

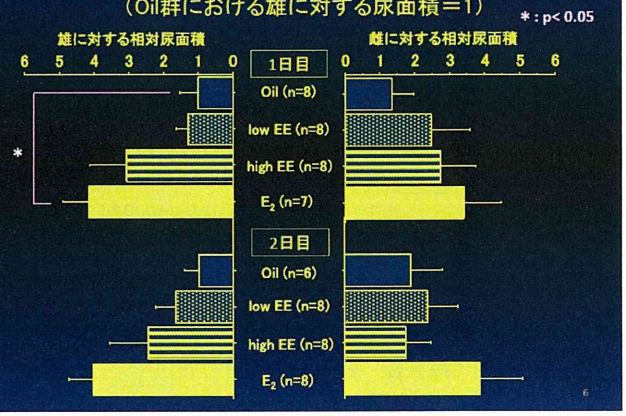


図7　EB投与時の受動回避学習試験結果

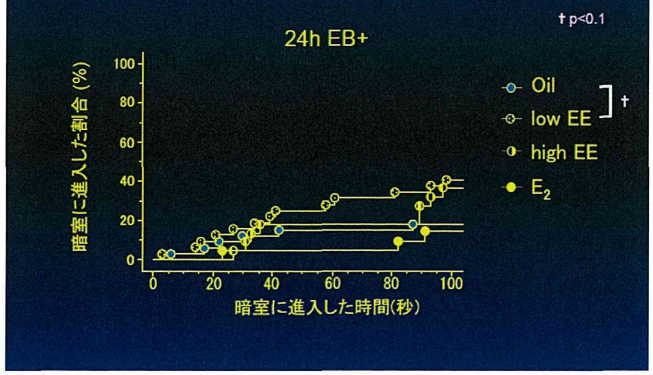


図8　EB非投与時の受動回避学習試験結果

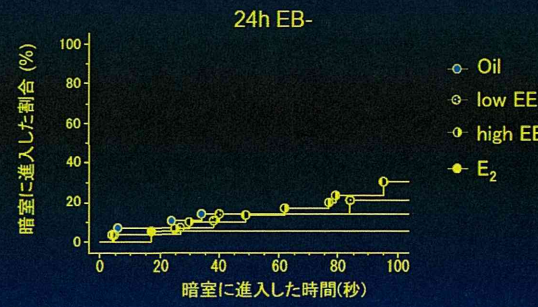


図9　海馬における成熟期ERα発現量

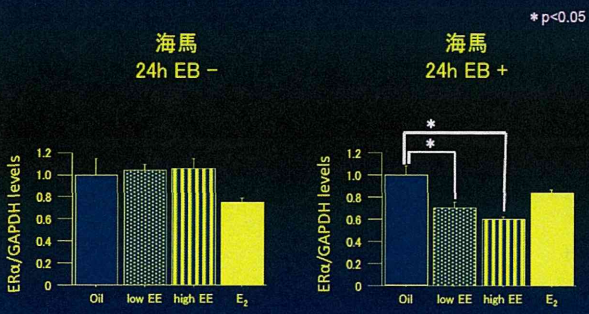


図10　海馬における成熟期ERβ発現量

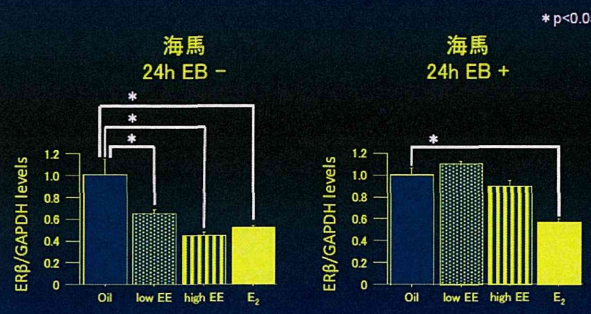


図11　大脳皮質における成熟期ERα発現量

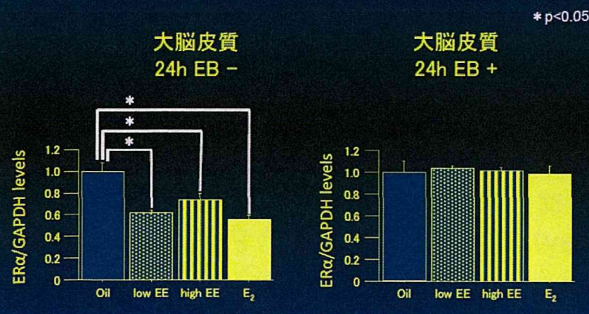
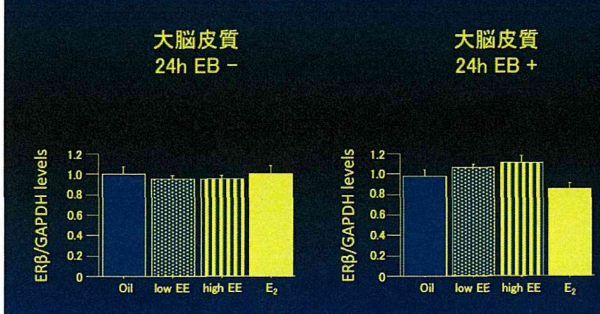
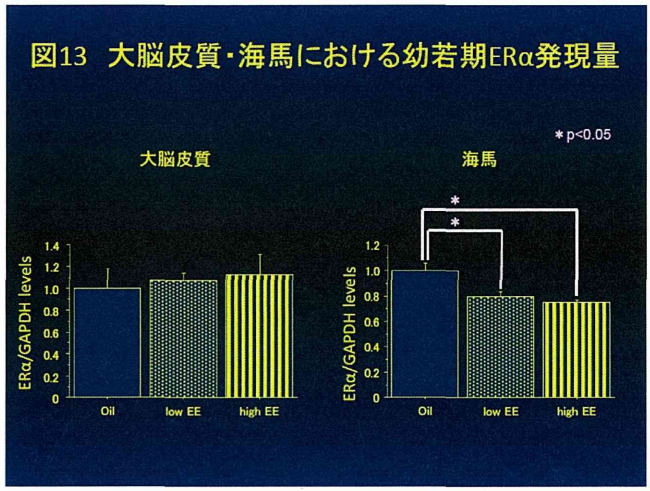
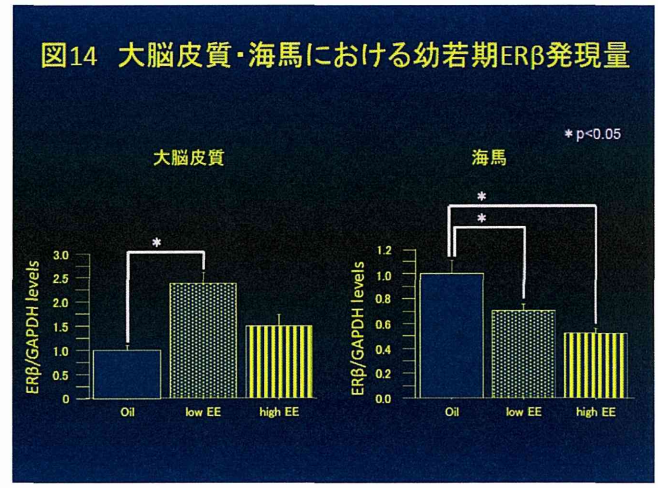


図12　大脳皮質における成熟期ERβ発現量







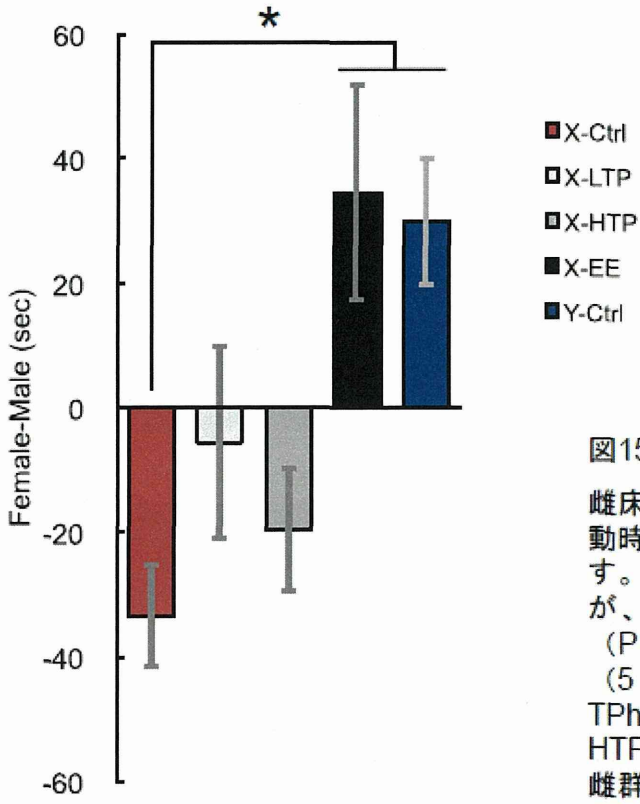


図15　12週齢時の性選好性試験の結果

雌床敷への接近行動時間と雄床敷接近行動時間への差を性選好性スコアとして示す。X-EE群とY-Ctrl群の性選好性スコアが、X-Ctrl群のスコアと比較して高い（$P < 0.05$）。図のX-CtrlはSesame oil（5 ml /kg /day）曝露雌群、X-LTPは低TPhP（25 mg /kg /day）曝露雌群、X-HTPは高TPhP（250 mg /kg /day）曝露雌群、X-EEはEE（15 μg /kg /day）曝露雌群、Y-CtrlはSesame oil（5 ml /kg /day）曝露雄群を示す。*: $P < 0.05$

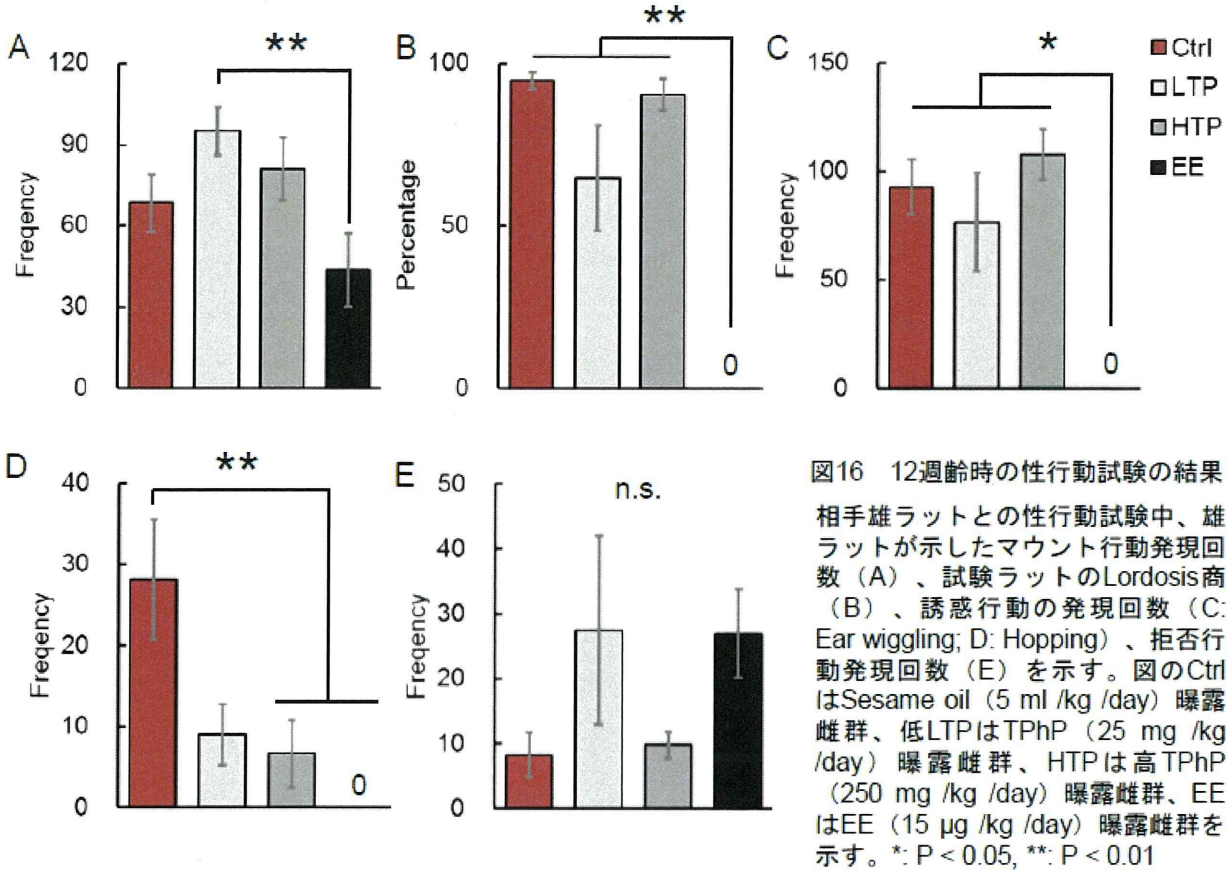


図16　12週齢時の性行動試験の結果

相手雄ラットとの性行動試験中、雄ラットが示したマウント行動発現回数（A）、試験ラットのLordosis商（B）、誘惑行動の発現回数（C: Ear wiggling; D: Hopping）、拒否行動発現回数（E）を示す。図のCtrlはSesame oil（5 ml /kg /day）曝露雌群、低LTPはTPhP（25 mg /kg /day）曝露雌群、HTPは高TPhP（250 mg /kg /day）曝露雌群、EEはEE（15 μg /kg /day）曝露雌群を示す。*: P < 0.05, **: P < 0.01

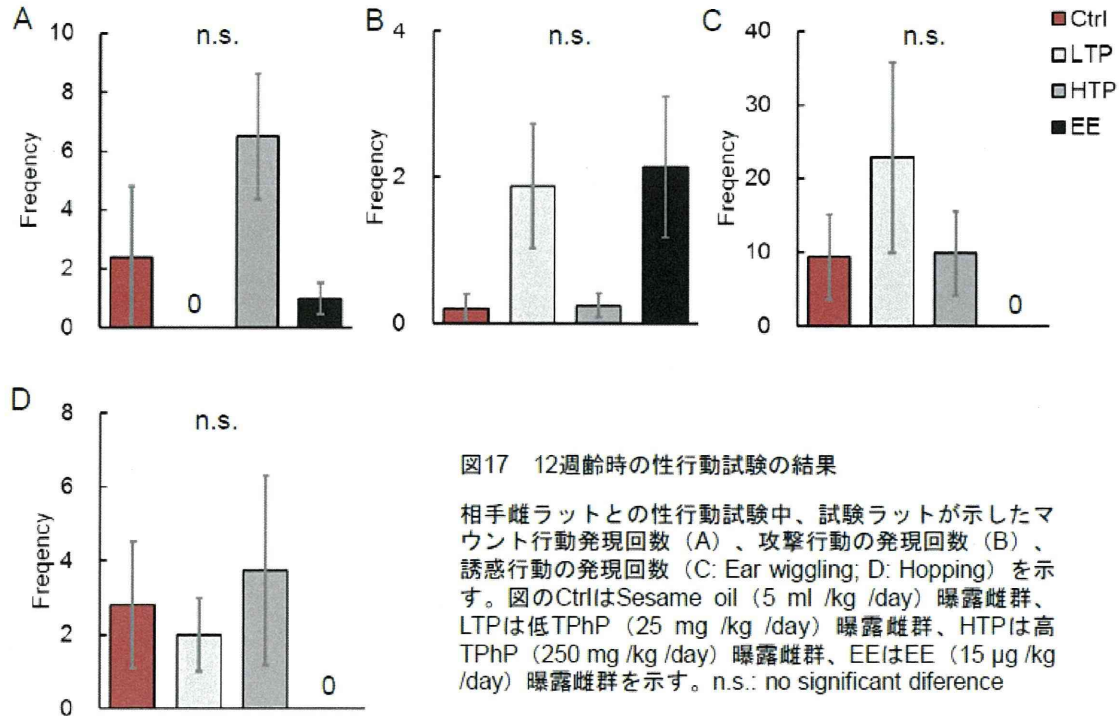


図17　12週齢時の性行動試験の結果

相手雌ラットとの性行動試験中、試験ラットが示したマウント行動発現回数（A）、攻撃行動の発現回数（B）、誘惑行動の発現回数（C: Ear wiggling; D: Hopping）を示す。図のCtrlはSesame oil（5 ml /kg /day）曝露雌群、LTPは低TPhP（25 mg /kg /day）曝露雌群、HTPは高TPhP（250 mg /kg /day）曝露雌群、EEはEE（15 μg /kg /day）曝露雌群を示す。n.s.: no significant diference

Ⅲ.研究成果の刊行に関する一覧表

雑誌

著者名	タイトル	雑誌名	巻・号・ページ	年
Takahashi M, Ichimura R, Inoue K, Morikawa T, Watanabe G, Yoshida M	The impact of neonatal exposure to 17alpha-ethynylestradiol on the development of kisspeptin neurons in female rats..	Repro Toxicol.,	60, 33-38	2016
Shiga T, Nakamura TJ, Komine C, Goto Y, Mizoguchi Y, Yoshida M, Kondo Y, Kawaguchi M	A Single Neonatal Injection of Ethinyl Estradiol Impairs Passive Avoidance Learning and Reduces Expression of Estrogen Receptor α in the Hippocampus and Cortex of Adult Female Rats.	PLoS One	11(1):e0146136.	2016
Ichimura R, Takahashi M, Morikawa T, Inoue K, Kuwata K, Usuda K, Yokosuka M, Watanabe G, Yoshida M.	The Critical Hormone-Sensitive Window for the Development of Delayed Effects Extends to 10 Days after Birth in Female Rats Postnatally Exposed to 17alpha-Ethynylestradiol. Biol Reprod., 93, 32, 2015.	Biol Reprod., 93, 32, 2015.	93, 32	2015.
Ichimura R, Takahashi M, Morikawa T, Inoue K, Maeda J, Usuda K, Yokosuka M, Watanabe G, Yoshida M.	Prior attenuation of KiSS1/GPR54 signaling in the anteroventral periventricular nucleus is a trigger for the delayed effect induced by neonatal exposure to 17alpha-ethynylestradiol in female rats.	Reproductive Toxicol	51:145-56.	2015
Yoshida M, Katashima S, Takahashi M, Ichimura R, Inoue K, Taya K, Watanabe G.	Predominant role of the hypothalamic-pituitary axis, not the ovary, in different types of abnormal cycle induction by postnatal exposure to high dose p-tert-octylphenol in rats.	Reprod Toxicol.	57, 21-28	2015.
Shiorta M, Kawashima J, Nakamura T, Kamiie J, Shirota K, Yoshida M.	Dose-dependent acceleration in the delayed effects of neonatal oral exposure to low-dose 17α-ethynylestradiol on reproductive functions in female Sprague–Dawley rats.	Journal of Toxicological Sciences	40, 727-738	2015
Taketa Y, Inoue K, Takahashi M, Sakamoto Y, Watanabe G, Taya K, Yoshida M.	Effects of sulpiride and ethylene glycol monomethyl ether on endometrial carcinogenicity in Donryu rats.	J Appl Toxicol.	Online Jul 14	2015

Horii Y, Kawaguchi M.	Higher detection sensitivity of anxiolytic effects of diazepam by ledge-free open arm with opaque walled closed arm elevated plus maze in male rats.	Behav Brain Res	294:131-40	2015
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Matsuo S, Takahashi M, Inoue K, Tamura K, Irie K, Kodama Y, Nishikawa A, Yoshida M.	Inhibitory Potential of Postnatal Treatment with Cyclopamine, a Hedgehog Signaling Inhibitor, on Medulloblastoma Development in Ptch1 Heterozygous Mice.	Toxicol Pathol.	42(8):1174-87	2014.
Nozawa K Nagaoka K, Zhang H, Usuda K, Okazaki S, Taya K, Yoshida M, Watanabe G.	Neonatal exposure to 17α-ethynyl estradiol affects ovarian gene expression and disrupts reproductive cycles in female rats,	Reprod Toxicology,	46, 77-84	2014.
Usuda K, Nagaoka K, Nozawa K, Zhang H, Taya K, Yoshida M, Watanabe G.	Neonatal exposure to 17α-ethinyl estradiol affects kisspeptin expression and LH-surge level in female rats	The Journal of Veterinary Medical Science	76, 1105-1110,	2014.
Hayashi S, Taketa Y, Inoue K, Takahashi M, Matsuo S, Irie K, Watanabe G, Yoshida M.	Effects of pyperonyl butoxide on the female reproductive tract in rats.	J Toxicol Sci.	38(6):891-902.	2013
Takahashi M, Inoue K, Morikawa T, Matsuo S, Hayashi S, Tamura K, Watanabe G, Taya K, Yoshida M.	Delayed effects of neonatal exposure to 17alpha-ethynylestradiol on the estrous cycle and uterine carcinogenesis in Wistar Hannover GALAS rats.	Reprod Toxicol.,	40, 16-23	2013
Matsuo S, Takahashi M, Inoue K, Tamura K, Irie K, Kodama Y, Nishikawa A, Yoshida M.	Thickened area of external granular layer and Ki-67 positive focus are early events of medulloblastoma in Ptch1$^{+/-}$ mice.	Exp Toxicol Pathol	65, 863-73	2013.
Shirota M, Kawashima J, Nakamura T, Ogawa Y, Kamiie J, Shirota K.	Vascular Hamartoma in the Uterus of a Female Sprague-Dawley Rat with an Episode of Vaginal Bleeding	Toxicologic Pathology	41, 1011-1015	2013

Ⅳ. 研究成果の刊行物

Reproductive Toxicology 60 (2016) 33–38

Contents lists available at ScienceDirect

Reproductive Toxicology

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

The impact of neonatal exposure to 17alpha-ethynylestradiol on the development of kisspeptin neurons in female rats

Miwa Takahashi[a,*], Ryohei Ichimura[a,b], Kaoru Inoue[a], Tomomi Morikawa[a], Gen Watanabe[b], Midori Yoshida[a]

[a] Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[b] Laboratory of Veterinary Physiology, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

ARTICLE INFO

Article history:
Received 29 May 2015
Received in revised form 12 January 2016
Accepted 22 January 2016
Available online 25 January 2016

Keywords:
Delayed effects
Neonatal exposure
17alpha-ethynylestradiol
Kiss1
AVPV

ABSTRACT

Neonatal exposure to 17alpha-ethynylestradiol (EE) at relatively low doses leads to delayed effects characterized by the early onset of age-related anovulation. Kisspeptin neurons in the anteroventral periventricular nucleus (AVPV), located at the anterior hypothalamus, are proposed to play key roles in appearance of these delayed effects after maturation. To understand the initial changes, we investigated Kiss1 mRNA expression in the anterior and posterior hypothalamus before weaning in female rats that received neonatal exposure to EE at various doses (0.002–2000 μg/kg). The level of Kiss1 mRNA in the anterior hypothalamus was decreased from 0.002 μg/kg which did not induce delayed effects. In the posterior hypothalamus, Kiss1 mRNA expression did not differ among the groups except 2000 μg/kg group. These results suggest that neonatal exposure to EE affects the development of kisspeptin neurons and kisspeptin neurons in the AVPV are highly susceptible to neonatal EE treatment.

1. Introduction

Exposure to chemicals with estrogenic activity during the critical time for brain sex differentiation (late embryonic to early postnatal stages in rodents) is known to cause irreversible reproductive deficits [1,2]. At a high dose, defeminization effects characterized by masculinized sexual behavior, lower gonadotropin levels during puberty, malformation of the reproductive tract, and cessation of cyclic ovulation occurs during the pre- or peri-pubertal periods [3,4]. In cases of low dose exposure, increased carcinogenic risk and impaired reproductive function can be apparent later in life in rodents as well as in humans, even though normal development occurs through maturation [5–7]. These are regarded as delayed effects, as distinguished from defeminization which occurs earlier. For chemical risk assessment, delayed effects have become a serious issue as they might be overlooked in existing reproductive toxicity or developmental toxicity studies in accordance with current authorized guidelines which only require limited observation periods.

We have previously investigated estrous cyclicity in rats that received a single injection of 17α-ethynylestradiol (EE) at dose levels of 0.02–200 μg/kg during the neonatal period [8]. In this study, although the vaginal opening was not affected, early onset of age-related anovulation was induced in a dose-dependent fashion after sexual maturation at 0.2 μg/kg of EE or more, and was considered a delayed effect. Although estrous cyclicity was regarded as a very useful indicator of delayed toxic effects on the female reproductive tract, which clearly demonstrated age- and dose-dependent effects, it takes a protracted time to detect the effects caused by neonatal exposure to estrogenic compounds [8]. Thus, toxicologic indicators applicable to early detection of delayed adverse effects are required for risk assessment of offspring toxicity.

From these view point, we have examined the changes occurring prior to abnormal estrous cycle, to find early indicators for subsequent delayed effects. As a result, we found that decreased expression of Kiss1 mRNA (encoding kisspeptin) in the anteroventral periventricular nucleus (AVPV) as well as concurrent depression of LH surges preceded the onset of abnormal estrous cycling [9]. In the arcuate nucleus (ARC), however, Kiss1 expression was not changed [9]. The AVPV is involved in control of the female estrous cycle [10], and kisspeptin neurons in the AVPV are presumed to be key players in the onset of these delayed effects. Significant reductions in Kiss1 mRNA expression in the hypothala-

* Corresponding author. Fax: +81 3 3700 1425.
E-mail address: mtakahashi@nihs.go.jp (M. Takahashi).

http://dx.doi.org/10.1016/j.reprotox.2016.01.008
0890-6238/

mus (including both the AVPV and ARC) were observed in rats that received neonatal injections of EE at postnatal day (PND) 14, even at the low dose of 0.02 μg/kg which was not sufficient to induce delayed effects [11]. Therefore, it is possible that neonatal exposure to EE might have an impact on kisspeptin neurons from very low dose before weaning. Given that the expression pattern of Kiss1 during the postnatal period has been reported to differ between the AVPV and ARC [12], region-specific analysis of Kiss1 during the developmental period would provide valuable insight into this process.

In this study, to clarify the initial changes leading to the delayed effects induced by neonatal exposure to EE, we investigated Kiss1 mRNA expression in the anterior and posterior hypothalamus before weaning, and examined the potential of Kiss1 as an early indicator for toxicological evaluation of delayed effects.

2. Materials and methods

2.1. Animals and chemicals

A total of 26 pregnant Wistar Hannover GALAS rats were obtained from CLEA Japan, Inc. (Tokyo, Japan) at gestational day 14 ($n=9$) and gestational day 15 ($n=17$). The rats were housed individually in polycarbonate cages with wood chip bedding and maintained in an air conditioned animal room (temperature: 24 ± 1 °C; relative humidity: 55 ± 5%; 12 h light/dark cycle) with a basal diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) and tap water available ad libitum. After delivery, 24 litters were used for the experiment, excluding 2 dams in which the timing of delivery was too delayed for dosing. The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

EE was purchased from Sigma (CAS No. 57-63-6; St. Louis, MO, USA) with purity > 98%. EE was stirred into a small amount of sesame oil overnight then used after dilution. EE was selected because of its rapid excretion and lower binding affinity for α-fetoprotein in neonatal blood. We previously confirmed that EE injected into neonatal rats was distributed to the brain and mostly excreted within 24 h, indicating that exposure time to EE is limited to several hours on PND0-1 [8].

2.2. Experimental design

To lessen the genetic difference between litters, pups born on the same day were collected and randomized within 24 h after birth. Then, 8 pups per dam (with a female predominance) were allocated to foster dams. Dams were assigned to 6 groups (4 dams/group), and all pups received a single subcutaneous injection of EE. The doses of EE were set based on our previous study [8] as follows: doses which did not induce delayed effects (no-effect level, 0.002 and 0.02 μg/kg body weight), doses which did induce delayed effects (delayed effect level, 0.2 and 20 μg/kg body weight) and a dose that leads to defeminization (2000 μg/kg body weight). The pups of the control group were injected with sesame oil (5 mL/kg body weight) as a vehicle.

On PND12, 14 and 21, 5 female pups per group were autopsied after measurement of body weight. The age at autopsy was determined based on the timing of follicle-stimulating hormone (FSH) secretion and Kiss1 mRNA expression during postnatal stages [11,13,14]. The animals were decapitated, and blood samples were collected for hormone assays. At PND14 and 21, the pituitary, ovaries, uterus, vagina and mammary glands were removed and fixed in 10% neutral buffered formalin. The weights of the ovaries and uteri were measured after fixation. These tissues were routinely processed and sectioned for hematoxylin and eosin (HE) staining. The intact uterine horns were cut into cross-section at 3 mm intervals. To elucidate the development of uterine glands, the number of uterine glands located away from the endometrium was counted, and the number of uterine glands per section per animal was calculated by dividing by the number of sections analyzed.

The brains were removed from the skulls, and the hypothalami were dissected out as described in a previous report [9]. A horizontal cut about 2 mm in depth was made with the following boundaries: 1 mm anteriorly from the optic chiasm, the posterior border of the mammillary bodies, and the hypothalamic fissures. Dissected hypothalami were macroscopically divided using the optic chiasm as a boundary into the anterior and posterior hypothalamus, each containing the AVPV and ARC. We had previously confirmed that the expression of Kiss1 mRNA in the anterior and posterior hypothalamus was equivalent to that in the AVPV and ARC, respectively [9]. Hypothalamic samples were immediately removed upon decapitation at PND14 and 21, and frozen in liquid nitrogen, then stored at −80 °C until processing for RNA analysis. The hypothalami from male rats of the control group were also collected at the same time for reference.

2.3. Hormone assays

Serum samples obtained after decapitation were stored at −80 °C until ready for assessment. Serum concentrations of FSH and luteinizing hormone (LH) were determined using double-antibody radioimmunoassays and ^{125}I-labeled radio-ligands. National Digestive and Kidney Disease (NIDDK) radioimmunoassay kits were used (NIAMDD, NIH, Bethesda, MD, USA) as described previously [15].

2.4. Real-time RT-PCR for Kiss1

Total RNA was isolated from the anterior and posterior hypothalamus using ISOGEN (NIPPON GENE Co., Ltd., Tokyo), and reverse transcription reactions were performed using 2 μg of total RNA with High Capacity Reverse Transcription kits (Applied Biosystems Japan Ltd., Tokyo, Japan). Following the manufacturer's instructions, real-time PCR was performed with an ABI Prism 7900HT (Applied Biosystems Japan Ltd.). Taqman® Gene Expression Assays (Applied Biosystems Japan Ltd.) were used to measure mRNA levels of Kiss1 metastasis-suppressor (Kiss1, Rn00710914_m1). The expression level of Kiss1 gene was calculated using the relative standard curve method and normalized against endogenous GAPDH (TaqMan Rodent GAPDH Control Reagent, Applied Biosystems Japan Ltd.). The expression level in the anterior hypothalamus of the control group at PND14 was expressed as 1, and relative levels were calculated for the other groups.

2.5. Statistical analysis

Following Bartlett's test, variance in data for body and organ weights, the number of uterine glands, hormone assays and real-time RT-PCR were compared to the control group by one-way analysis of variance or the Kruskal–Wallis test. When statistically significant differences were detected, Dunnett's multiple comparison test was employed for comparison between the control group and the treatment groups. The mRNA expression levels in males were compared using Student's t-test following a test for equal variance.

3. Results

3.1. Mortality and body growth

One animal in each of the control and 2000 μg/kg group died before PND7. No other deaths or abnormal clinical signs were found

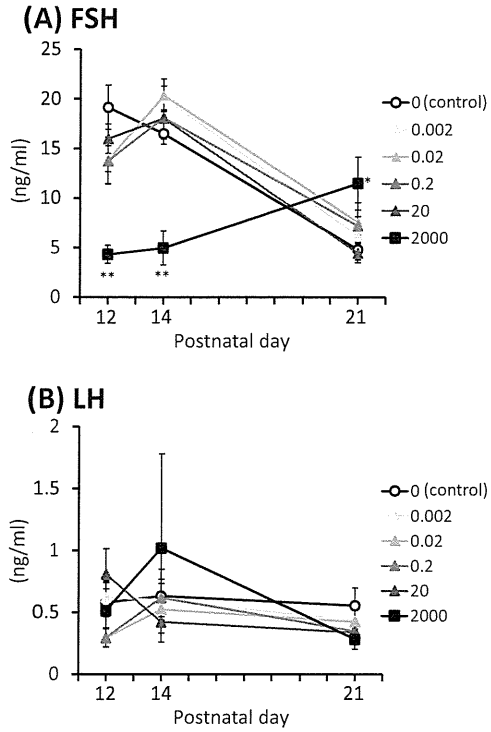

Fig. 1. Serum FSH (A) and LH (B) levels at postnatal day 12, 14 and 21 in female rats that received a single injection of EE. Data represent the mean ± SEM. $n = 5$ per group. *, **: Significantly different from the 0 μg/kg group at $p < 0.05$ and 0.01, respectively (Dunnet's test).

through the end of the study. The average body weight of female pups did not differ among the groups during the experimental period. Although statistical differences were occasionally found in the body weights of animals autopsied at PND12, 14 and 21, these were regarded as incidental because there was no correspondence to dose level (data not shown).

3.2. Serum FSH and LH level

The level of serum FSH in the control group was highest at PND12, then gradually decreased at PND14 and 21 (Fig. 1). In the groups of no-effect level (0.002 and 0.02 μg/kg) and delayed effect level (0.2 and 20 μg/kg), serum FSH levels were not significantly different from the control group, although peak FSH expression in these groups was shifted to PND14. In contrast, the FSH level in the defeminization group (2000 μg/kg) was remarkably lowered at PND12 and 14, and was significantly elevated at PND21 compared to the other groups. The level of serum LH showed large variation due to individual difference, and there were no intergroup differences in serum LH levels at all time points examined.

3.3. Development of female reproductive organs

Weights of the ovaries and uterus at PND14 and 21 are shown in Fig. 2. At PND14, weights of the ovaries and uterus tended to decrease in a dose-dependent manner, whereas such a tendency was not obvious at PND21. In the 2000 μg/kg group, absolute and relative weights of the ovaries and uterus were significantly lowered compared to the control group at PND14, and a similar tendency was found at PND21. Among the dosing groups that induced delayed effects, a significant reduction in the relative weight of the ovaries and uterus was seen in the 20 μg/kg group at PND14. However, organ weights of these groups were comparable to those of

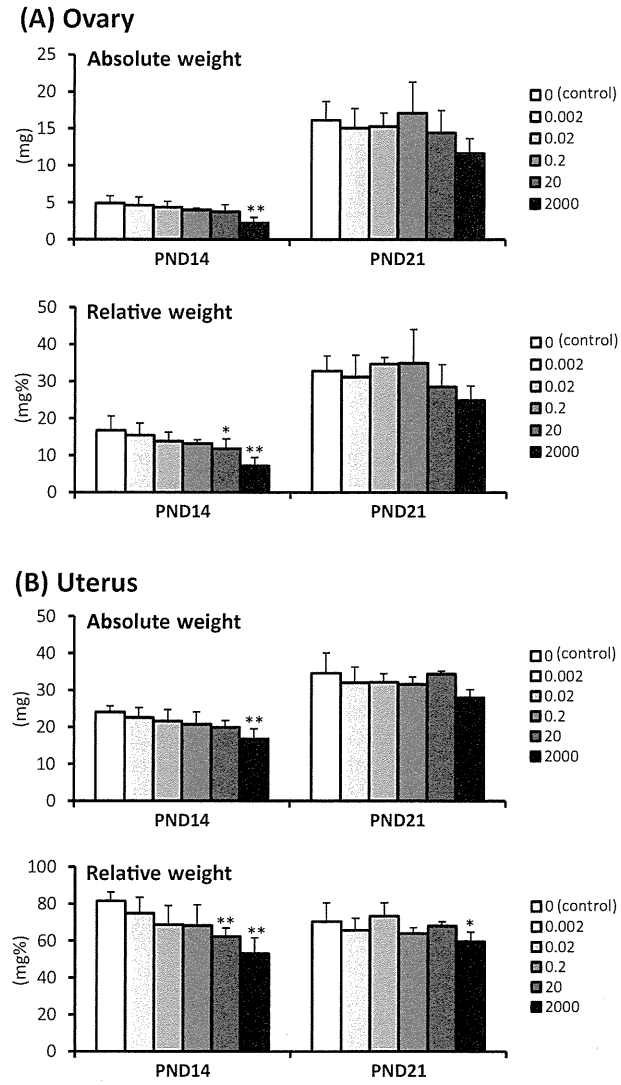

Fig. 2. Weights of the ovaries (A) and uterus (B) at postnatal day 14 and 21 in female rats that received a single injection of EE. Data represent the mean ± SD. $n = 5$ per group. *, **, Significantly different from the 0 μg/kg group at $p < 0.05$ and 0.01, respectively (Dunnet's test).

the control group at PND21. There were no intergroup differences in organ weight between the control group and the no-effect groups.

Histopathological examination of the ovaries demonstrated that the folliculogenesis stage extended to the small antral follicle at PND14 and had advanced to the large antral follicle by PND21. There were no polyovular follicles in the control group at PND14 and 21. In the EE-treated groups, polyovular follicles were found in 1–3 of 5 animals, but without dose-dependency. There were no apparent abnormalities in uterine histology in any group. The number of the uterine glands per section at PND14 was similar among all groups, although there was a slight increase in number in the 2000 μg/kg group (Fig. 3). At PND21, there was a decreasing tendency or significant decrease in the number of uterine glands found in the EE treated groups except for the 0.002 μg/kg group.

During normal development at PND14 and 21, the vaginal mucosa was composed of 3–4 layers of non-keratinized cells (Fig. 4). Only in the 2000 μg/kg group, keratinized eosinophilic cells and mucinous cells were observed in the vaginal mucosa at PND14, similar to rats neonatally treated with diethylstilbestrol [16]. The mucosa was normal in all other groups.

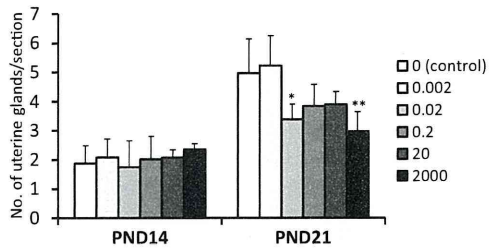

Fig. 3. Number of uterine glands per section at postnatal day 14 and 21. Data represent the mean ± SD. $n = 5$ per group. *, **, Significantly different from the 0 μg/kg group at $p < 0.05$ and 0.01, respectively (Dunnet's test).

3.4. Kiss1 mRNA expression

The expression of Kiss1 mRNA in the anterior and posterior hypothalamus is shown in Fig. 5. The relative expression levels based on sex, age and regions of the hypothalamus in females and males in the control group were consistent with previous findings [12]. In the 2000 μg/kg group, Kiss1 mRNA expression in the anterior hypothalamus was remarkably lowered at both PND14 and 21, compared to the control group. Moreover, in the posterior hypothalamus, the level of Kiss1 showed a tendency to decrease at PND14, and resembled males in the expression pattern. Kiss1 mRNA in the anterior hypothalamus in the groups of no-effect and delayed effect levels was equally decreased at PND14. The dose-dependency was not clear, and statistical significance was not detected due to the large variation in the control group. Significant reductions in Kiss1 mRNA were also found at PND21 in these groups; however, the gap with the control group tended to reduce compared to that at PND14. Expression of Kiss1 in the posterior hypothalamus of the groups that induced no effect or the groups that induced delayed effects did not differ from the control group at PND14 and 21.

4. Discussion

Our results demonstrate that the development of kisspeptin neurons could be affected by neonatal exposure to EE. In particular, the expression of Kiss1 mRNA in the anterior hypothalamus

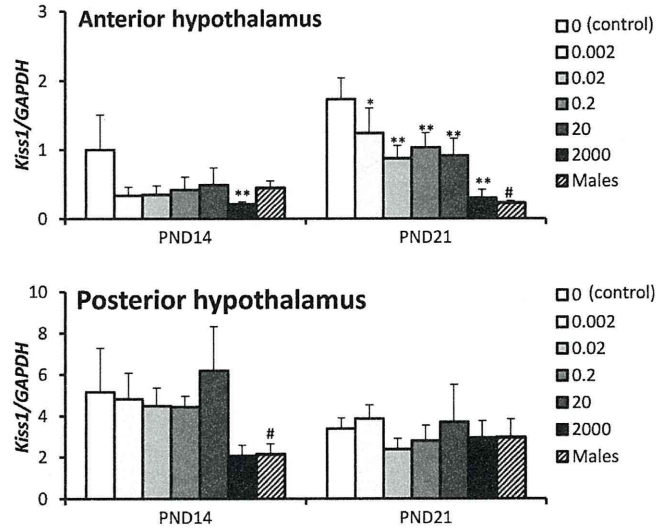

Fig. 5. Kiss1 expression in the anterior and posterior hypothalamus at postnatal day 14 and 21. Data represent the mean ± SD. $n = 5$ per group. *, **, Significantly different from the 0 μg/kg group at $p < 0.05$ and 0.01, respectively (Dunnet's test). #: $p < 0.05$ vs. 0 μg/kg group (Student's t-test).

was decreased even at the lowest doses which did not induce delayed effects, indicating that kisspeptin neurons in the AVPV have high susceptibility to EE. In contrast, Kiss1 levels in the posterior hypothalamus were lowered only in the dose group that caused defeminization, but was not affected in the groups with no-effect or groups that induced delayed effects. These results suggest that the ARC is less susceptible to EE than the AVPV.

The AVPV and ARC are both sexually dimorphic brain regions in which females have a larger number of neurons and higher cell density than males [12]. Experimental studies have reported that kisspeptin neurons are sensitive to sex steroids during the critical window of brain sex differentiation, the perinatal period in rodents [17]. Exposure to high doses of testosterone or estrogenic compounds during this period in female rats induces masculinization of the AVPV and ARC [18]. Based on studies which used agonists selective for estrogen receptor (ER) as well as knockout mouse

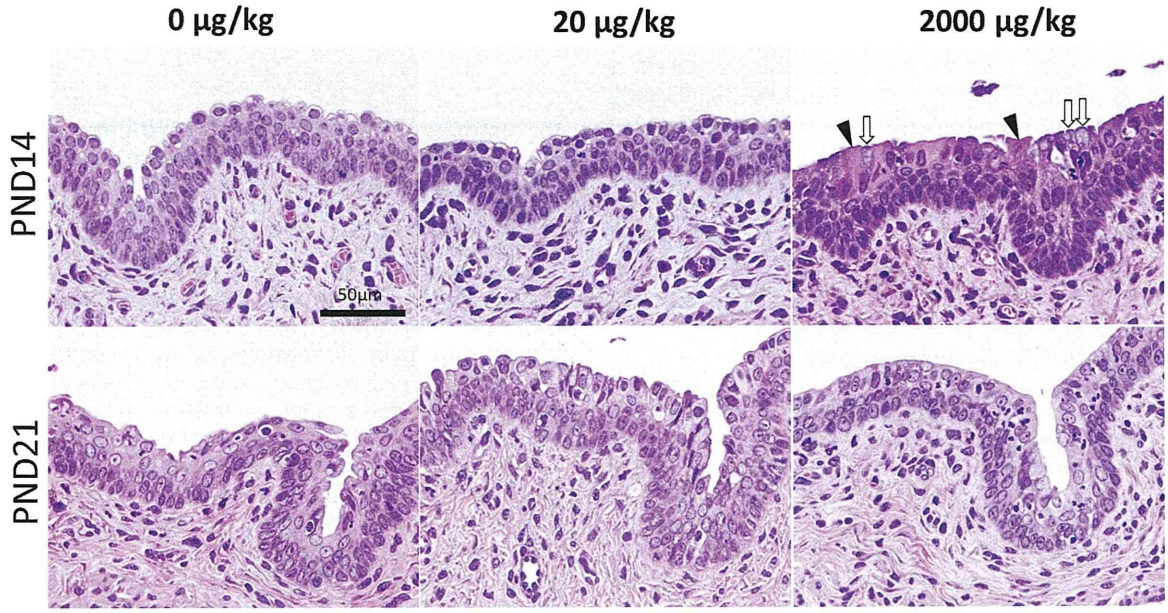

Fig. 4. Histology of the vaginal mucosa at postnatal day 14 and 21. Eosinophilic keratinized cells (black arrowhead) and mucinous cells (white arrow) were observed in the vaginal mucosa only in the 2000 μg/kg group at PND14. HE stain. Scale bar: 50 μm.

models, it is suggested that ERα plays a pivotal role in the sexual differentiation of Kiss1 neurons in rodents [19,20]. In rats, it has been reported that expression of Kiss1 mRNA in the AVPV is first detected around PND10 then rapidly increases in females [12,21]. Detectable level of Kiss1 mRNA was found in the ARC from PND0 in both sexes [12,21]. ERα mRNA was detected by in situ hybridization in both the AVPV and ARC from PND0, and the expression level was reported to be higher in the AVPV than in the ARC [21]. This might partly explain why the AVPV shows high susceptibility to EE during the neonatal period, although the precise mechanism by which neonatal estrogen affects the expression of Kiss1 more than one week later remains unclear.

The expression pattern of Kiss1 in defeminized rats was clearly distinguished from that of the other groups. At the defeminization dose level, suppressed Kiss1 mRNA expression in the anterior hypothalamus was maintained from PND14 to 21. Moreover, Kiss1 mRNA expression in the posterior hypothalamus was lowered to the same level as in males. In contrast, although Kiss1 expression in the groups with either no effects or delayed effects was decreased at PND14 and 21 compared to the control group, the expression level of these groups indicated a tendency toward the level of the control group at PND21. So far, it is well known that neonatal treatment of estrogen at high dose (i.e. deferminization level) exerted an inhibitory influence on Kiss1 gene expression. For example, female rats subjected to high-dose neonatal exposure to estradiol benzoate displayed a significant decrease in hypothalamic Kiss1 mRNA at 30 days of age [22]. Neonatally androgenized females exhibited fewer numbers of Kiss1 expressing cells in the AVPV than normal females in adulthood, similar to the male pattern [23]. In contrast, studies that investigate dose response and low dose effect of neonatal treatment of estrogen in females is very limited. Our previous study showed that Kiss1 expression in female rats that received a single injection of EE up to 200 μg/kg during the neonatal period was comparable between the control and EE-treated groups at 5 or 10 weeks of age [11]. Accordingly, within the dose range for not inducing defeminization, it is thought that Kiss1 mRNA in the AVPV decreases temporarily and the gap between the control and EE-treated groups would be reduced in conjunction with sexual maturation. Although the precise mechanism for recovery of Kiss1 expression around puberty is unknown and more detailed analysis during development and peripubertal period is needed, occurrence of normal puberty is crucial to allow the effect of neonatal exposure after sexual maturation, as delayed effects. The threshold of defeminization induced by neonatal EE injection exists between 200 and 2000 μg/kg based on our previous and present studies [11].

In female rats, it is known that serum FSH concentration is temporarily elevated around PND10-15 through positive feedback by estradiol [13,14]. It has been reported that serum FSH levels are reduced in androgenized female rats during the prepubertal period, and this reduction is accompanied by lower hypothalamic levels of LH releasing factor [24,25]. Similar to previously reported data, lowered FSH was observed in the 2000 μg/kg group in our study. This might be attributed to diminished estrogen positive feedback caused by suppression of Kiss1 expression, although serum estrogen levels were not measured in this study. Although it has been reported that LH level reaches maximum around PND15 [14], such a pattern of LH level was not clear in this study. LH is known to show large individual difference due to diurnal fluctuations in prepubertal rats [26], and that might disturb showing intergroup differences. Consequently, it is thought that decrease in the ovarian weight is due to lowered FSH secretion, and the inhibition of uterine gland genesis and histological changes observed in the vagina at PND14 are considered to be secondary effects due to impairment of the hypothalamic-pituitary-gonadal axis [4,27].

From a risk assessment perspective, there are concerns that any delayed effects might be overlooked by existing toxicity studies due to the unique onset of the adverse effects. Although we proposed that estrous cyclicity is a precise indicator of delayed effects in the female reproductive tract [8], it takes a protracted amount of time to detect the effects caused by neonatal exposure to estrogenic compounds. Early indicators capable of predicting delayed effects are thus required, hence we examined the validity of some potential parameters for detection.

A significant decrease in Kiss1 mRNA expression in the whole hypothalamus was observed at PND14 in rats neonatally exposed to EE even at the no-effect dose level [11]. Decreased expression in Kiss1 mRNA in the anterior hypothalamus at PND14 was obvious in the EE-treated groups in the present study, and it was confirmed that AVPV was the target of EE by region-specific analysis. Additionally, we reported that decreased expression of Kiss1 mRNA in the AVPV and depression of the LH surge occur prior to the onset of abnormal estrous cycling, indicating that kisspeptin neurons in the AVPV that control ovulation play a key role in these delayed effects [9]. Thus, reductions in Kiss1 mRNA expression at PND14 and 21 might possibly be implicated in the impaired function of kisspeptin neurons after sexual maturation. In contrast, dose-dependency in Kiss1 mRNA level at PND14 was unclear in both studies, even though estrous cyclicity clearly demonstrated dose-dependent effects of neonatal EE exposure. Decreased expression of Kiss1 mRNA was found in the dose groups which had no effects as well as in the groups that showed delayed effects. Therefore, there are possibilities that decreased expression of Kiss1 mRNA before weaning is not directly linked to early onset of anovulation or other factors in addition to Kiss1 might also be required for the onset of these delayed effects after maturation. Further investigation is needed to clarify whether this is the only event of the beginning and directly linked to early onset of anovulation, and to confirm whether decreased Kiss1 mRNA expression occurs by chemicals inducing delayed effects other than EE.

In our previous study, lowered FSH levels at PND14 were found in animals neonatally exposed to EE at 2 μg/kg or higher [11]. The present results showed that the level of FSH did not significantly differ among the groups, except for the defeminized group, although the peak day was slightly shifted. Accordingly, it is suggested that the FSH level is characteristically lowered at defeminizing dose levels of EE, whereas FSH is maintained within a normal range at EE doses resulting in delayed effects. Although polyovular follicles were found only in the EE-treated groups in this study, polyovular follicles were observed even in the control group in our previous study [11]. Since there was no correlation between the occurrence of polyovular follicles and dosage level in both our previous and present study, we considered that polyovular follicles were unrelated to neonatal EE exposure. In rats, neonatal treatment with estrogenic compounds was reported to induce slightly premature gland genesis but subsequently lowered the number of uterine glands [28,29]; similar results were observed in the defeminization group in the present study. A lowered number of uterine glands at PND21 was also found in both our previous and present studies [11]. However, the dose that induces suppression of uterine gland development varied between the two studies, thus this finding was regarded to be unsuitable for prediction of delayed effects.

5. Conclusions

Our results suggest that neonatal exposure to EE affects the development of kisspeptin neurons, especially in the AVPV, and this results in decreased expression of Kiss1 mRNA before weaning. At relatively low EE dose levels, the decrease in Kiss1 expression might be temporary and results in normal sexual maturation, distinguishing this effect from defeminization. Further examination is required to demonstrate that decrease in Kiss1 mRNA in the AVPV

before weaning directly lead to early onset of anovulation and its utility as a parameter for detection of delayed adverse effects.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

We thank Mss. Ayako Saikawa and Yoshimi Komatsu for technical assistance in conducting the animal study. This study was supported by Health and Labour Sciences Research Grants, Research on Risk of Chemical Substances, Ministry of Health, Labour and Welfare, Japan [H25-Toxicol-003].

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RESEARCH ARTICLE

A Single Neonatal Injection of Ethinyl Estradiol Impairs Passive Avoidance Learning and Reduces Expression of Estrogen Receptor α in the Hippocampus and Cortex of Adult Female Rats

Tatsuomi Shiga[1], Takahiro J. Nakamura[1,2], Chiaki Komine[1], Yoshikuni Goto[2], Yasushi Mizoguchi[1], Midori Yoshida[3], Yasuhiko Kondo[4], Maiko Kawaguchi[1]*

1 School of Agriculture, Meiji University, Kawasaki, Kanagawa, Japan, **2** Faculty of Pharmaceutical Sciences, Teikyo Heisei University, Tokyo, Japan, **3** Division of Pathology, National Institute of Health Sciences, Tokyo, Japan, **4** School of Life and Environmental Sciences, Teikyo University of Science, Tokyo, Japan

* maiko@meiji.ac.jp

OPEN ACCESS

Citation: Shiga T, Nakamura TJ, Komine C, Goto Y, Mizoguchi Y, Yoshida M, et al. (2016) A Single Neonatal Injection of Ethinyl Estradiol Impairs Passive Avoidance Learning and Reduces Expression of Estrogen Receptor α in the Hippocampus and Cortex of Adult Female Rats. PLoS ONE 11(1): e0146136. doi:10.1371/journal.pone.0146136

Editor: Marià Alemany, University of Barcelona, Faculty of Biology, SPAIN

Received: June 6, 2015

Accepted: December 14, 2015

Published: January 7, 2016

Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported by Research on Risk of Chemical Substances, Health and Laboratory Sciences Research Grants, Ministry of Health, Labour and Welfare, Japan (H25-KAGAKU-IPPANN-003) to MK (http://www.mhlw.go.jp/english/index.html).

Abstract

Although perinatal exposure of female rats to estrogenic compounds produces irreversible changes in brain function, it is still unclear how the amount and timing of exposure to those substances affect learning function, or if exposure alters estrogen receptor α (ERα) expression in the hippocampus and cortex. In adult female rats, we investigated the effects of neonatal exposure to a model estrogenic compound, ethinyl estradiol (EE), on passive avoidance learning and ERα expression. Female Wistar-Imamichi rats were subcutaneously injected with oil, 0.02 mg/kg EE, 2 mg/kg EE, or 20 mg/kg 17β-estradiol within 24 h after birth. All females were tested for passive avoidance learning at the age of 6 weeks. Neonatal 0.02 mg/kg EE administration significantly disrupted passive avoidance compared with oil treatment in gonadally intact females. In a second experiment, another set of experimental females, treated as described above, was ovariectomized under pentobarbital anesthesia at 10 weeks of age. At 15–17 weeks of age, half of each group received a subcutaneous injection of 5 μg estradiol benzoate a day before the passive avoidance learning test. Passive avoidance learning behavior was impaired by the 0.02 mg/kg EE dose, but notably only in the estradiol benzoate-injected group. At 17–19 weeks of age, hippocampal and cortical samples were collected from rats with or without the 5 μg estradiol benzoate injection, and western blots used to determine ERα expression. A significant decrease in ERα expression was observed in the hippocampus of the estradiol-injected, neonatal EE-treated females. The results demonstrated that exposure to EE immediately after birth decreased learning ability in adult female rats, and that this may be at least partly mediated by the decreased expression of ERα in the hippocampus.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Xenoestrogens are compounds in the environment that mimic the physiologic activity of estrogen; they are contained in industrial contaminants, plastics or plasticizers, pesticides, and certain plants [1,2]. By binding to estrogen receptors (ERs), these compounds can disturb homeostatic responses in the endocrine system [1,3]. In fact, exposure to such estrogenic substances can have a profound adverse influence on the development of the nervous system in both animals and humans. One such influence is the impairment of learning and memory [4–9]. For example, the female offspring of Wistar rat dams exposed during pregnancy and lactation to bisphenol A (BPA), an estrogenic agent in polycarbonate plastics, demonstrated impaired learning in step-down passive avoidance tasks as adults [6]. Additionally, the female progeny of dams exposed from gestation to lactation to the estrogenic agent isobutylparaben, a widely used preservative, demonstrated poor social recognition performance [7]. Notably, the heavy metal pollutant cadmium can also mimic estrogens [10], such that cadmium toxicity can inhibit avoidance acquisition in female offspring [9].

The mechanism through which these toxic effects are induced may involve changes in hippocampal ERα expression after maturation. In female rodents, acute estrogen treatment enhanced hippocampus-dependent learning behaviors such as avoidance and spatial memory [11–13]. At the molecular level, however, little is known about the effect of perinatal xenoestrogen exposure on hippocampal or cortical ERα expression, although many reports have demonstrated altered ERα expression in the hypothalamus [14–16]. Kundakovica et al. demonstrated that exposure to 20 μg/kg BPA during lactation reduced ERα expression in the prefrontal cortex, but not in the hippocampus, in intact female mice [17]. BPA, however, also disturbs thyroid activity, so it remains unclear whether the reduced ERα expression was specifically induced by the estrogenic activity of BPA. In addition, neonatal exposure to estrogenic compounds can affect gonadal development and subsequent blood estrogen levels after maturation [18], and most studies cannot exclude this indirect effect on the brain and behavior. Therefore, there is a gap in our understanding of how the amount and timing of xenoestrogen exposure directly affects learning behavior and/or ERα expression in the hippocampus and cortex.

In this study, our objective was to determine whether a single neonatal dose of a xenoestrogen, and if so what dose, would directly affect learning behavior and ERα expression in the hippocampus and cortex. We selected ethinyl estradiol (EE), a constituent of contraceptives, as a model compound. Because EE does not bind to α–fetoprotein, it is transported to the brain and excreted from the body within 24 h after a subcutaneous injection, thus limiting its exposure period [19,20]. Rats were exposed to a low dose (0.02 mg/kg EE; LEE), that was chosen based on a study reporting early onset of persistent estrus from 14 weeks of age in rats that received a single neonatal injection of EE [20]; or a high dose (2 mg/kg EE; HEE) that was selected based on data we collected previously, in which sexual behavior in rats was inhibited by a single injection of EE (unpublished data, Maiko Kawaguchi). In addition, 20 mg/kg 17β-estradiol (E2) was chosen as a comparison based on a previous study reporting the loss of sexual differentiation of the sexually dimorphic nucleus of the preoptic area (POA) following E2 exposure [21]. For behavioral testing, we selected the passive avoidance test, which has been validated for estrogen sensitivity [11]. The passive avoidance test is also known to be affected by perinatal estrogenic agents in females [6], and has been shown to utilize anatomical substrates including the hippocampus [22,23], and its associated cortex [24]. In addition, the direct effects of EE were tested by ovariectomizing (OVX) one group of females and providing a controlled replacement does of estrogen to half the group.

Materials and Methods

Animals

Pregnant Wistar-Imamichi rats were obtained from the Institute for Animal Breeding Research (Ibaraki, Japan). Animals were maintained under controlled air conditions (room temperature [RT] 23 ± 1°C; humidity 50% ± 15%) with food and water available ad libitum, under a 12/12 h light/dark cycle with a light intensity of 200–300 lux. All procedures were approved by the Animal Care and Use Committee of Meiji University of Agriculture (approval ID#: IACUC11-0015).

Treatments and test schedule

Eight offspring per litter were selected within 24 h after birth. Subsequently, female pups were subcutaneously injected with one of the following: 0.02 mg/kg EE (Tokyo Chemical Industry, Tokyo, Japan; LEE), 2 mg/kg EE (HEE), 20 mg/kg E2 (Sigma-Aldrich, St. Louis, MO, USA), or vehicle only (sesame oil, Sigma-Aldrich). They were weaned at the age of 3 weeks. In the first experiment, at 6 weeks of age, the rats underwent the passive avoidance test while gonadally intact, regardless of their estrus stage. Because neonatal exposure to xenoestrogens is known to affect both the gonads and brain, we next generated groups designed to investigate the specific effects on the brain. For this, another set of animals was divided into the same neonatal injection groups described above, then surgically ovariectomized (OVX) and treated with or without estradiol benzoate (5 μg/0.1 ml; EB). The OVX with EB group modeled normal fertile females, while the OVX without EB group modeled menopause. Females of all groups were OVX under pentobarbital anesthesia (40 mg/kg) at 10 weeks of age. At 15–17 weeks of age, 24 h before the passive avoidance test, half of the OVX females from all injection groups were injected with 5 μg/0.1 ml EB (Sigma-Aldrich). Two weeks later, 24 h prior to brain sampling for western blotting, the same females were again injected with 5 μg/0.1 ml EB. Passive avoidance testing was performed in quadruplicate in both the OVX with and without EB groups (n = 6–10/group). Six OVX animals per group were randomly chosen after the first set of behavioral tests for subsequent brain tissue collection for western blotting.

Passive avoidance test

All rats were trained for the passive avoidance test. The step-through type passive avoidance test unit (PA-2010A & PAA-3001; O' Hara & Co., Tokyo, Japan) comprised two compartments: bright and dark (500 × 150 × 270 mm). An automated guillotine door was used to isolate the compartments. The passive avoidance test consisted of acquisition and testing phases. During acquisition, rats were placed in the bright compartment with the door opened. After the rat moved into the dark compartment (all rats voluntarily moved within 100 s), the door was closed to restrict the rats to the dark compartment. Then, they were given a mild electric shock (0.3 mA for 3 s) through the floor grid. Infrared sensors monitored movement from the bright compartment to the dark compartment, which was recorded as transfer latency time in seconds. The testing phase was carried out 24 h after the acquisition phase. Transfer latency was recorded, and if the rats did not enter the dark compartment within 100 s, this was recorded as "no response." The apparatus was cleaned with 70% ethanol solution before every test.

Brain sample collection

In the second experiment, we also examined the expression levels of ERα in the cortex and hippocampus of EE-treated OVX rats with or without EB. At 17–19 weeks of age, rats were

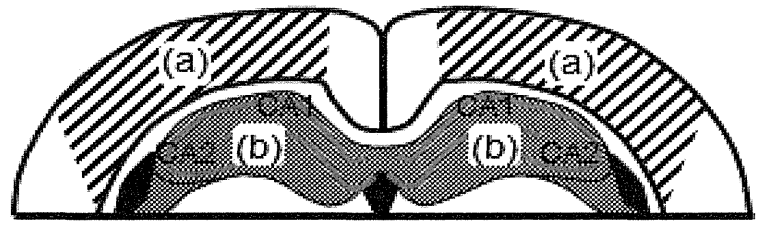

Fig 1. Illustration of a coronal section of the brain showing tissue sampling sites for the cortex (a) and hippocampus (b) [25]. CA1: CA1 region of the hippocampus; CA2: CA2 region of the hippocampus.

doi:10.1371/journal.pone.0146136.g001

sacrificed by deep ether anesthesia, and their brains removed. The brains were placed in chilled saline and sliced coronally at a thickness of 1 mm using a metal brain slicer (Muromachi, Tokyo, Japan). The cortex (Fig 1a) and hippocampus (Fig 1b) were isolated from the slice using scalpels under a stereomicroscope and stored at -80°C for processing. For homogenization, tissues were dispensed in 1 μl/mg lysis buffer containing mammalian protein extraction buffer (GE Healthcare, Connecticut, USA) and a 1% inhibitor cocktail (Thermo Scientific, Massachusetts, USA). Tissues were homogenized and sonicated on ice for 2 × 5 s each. Samples were centrifuged for 10 min at 14000 rpm at 4°C, and their supernatants were collected. The protein concentrations were determined using a 2-D Quant kit (GE Healthcare). The samples were mixed with a one-sixth volume of 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 2.5% SDS, and 0.01% bromophenol blue, boiled for 5 min, and stored at -80°C.

Western blotting

Frozen samples (40 μg protein) were separated on a 5–15% SDS-polyacrylamide gradient gel (BIO CRAFT, Tokyo, Japan) at 40 mA for 3 h. Molecular weight markers (Dual Color; BIO RAD, California, USA) were included in the run. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes overnight at 30 V. The membranes were blocked with 5% skim milk in TBS-T (100 mM Tris, 2.0% NaCl pH 7.5, 1% Tween-20) for 60 min at RT. The membranes were incubated overnight at 4°C with the ERα rabbit polyclonal antibody (MC-20, 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in TBS-T. Protein loading was normalized to GAPDH using a monoclonal primary antibody (6C5, 1:5000 dilution; Santa Cruz Biotechnology). The membranes were washed three times with TBS-T for 5 min each and then incubated with the anti-rabbit (W401B, 1:10000 dilution; Promega, Wisconsin, USA) or anti-mouse secondary antibody (W402, 1:10000 dilution; Promega) for 1 h at RT. Antibody staining was detected using the enhanced chemiluminescence kit (ECL prime; GE Healthcare). The signals in developed images were quantified using ImageJ software (NIH, USA). The results are expressed as intensity of the signals in arbitrary densitometry units after normalization to GAPDH as an internal standard. Western blot analyses were done separately for the EB (-) and EB (+) injected groups due to the equipment's limited sample capacity.

Statistical analysis

Results of the passive avoidance test were analyzed by Kaplan-Meier survival analysis, followed by log-rank comparison. For other measures, one-way analyses of variance (ANOVA) and *post-hoc* Tukey-Kramer tests were used to compare multiple groups. Results of the ANOVAs are presented as the mean ± SEM. All results were considered significant at $P < 0.05$.