厚生労働科学研究補助金（化学物質リスク研究事業）
分担研究報告書

化学物質の臨界期曝露による生殖内分泌機能のち初影響に視床下部キスペプチンニューロンの部位特異的変化が果たす役割と閾値に関する研究

分担研究課題：化学物質およびホルモンの臨界期曝露による神経内分泌系への遅発型影響に対する神経行動学的アプローチ

研究分担者： 川口真以子　学校法人明治大学　農学部　動物環境学研究室
研究協力者： 服部達哉　学校法人明治大学　研究・知財戦略機構

研究要旨（枠囲）

本研究は周生期にエストロゲン様作用をもつ化学物質（EDs）のうち17α-ethynyl estradiol（EE）およびTriphenyl phosphate（TPhP）を連続曝露し、雌ラットの発達と成長後の社会性行動発現への影響を明らかにすることを目的とした。その結果、EEは周産期連続曝露によって卵巣の発達を抑制し、成熟後の性行動中の雌特異的な社会性行動を抑制することを明らかにした。さらに、これまでin vivo条件下でのエストロゲン様作用が不明であったTPhPは、その曝露濃度依存的に成熟後の性行動をEEと同様に抑制する作用を持つことを明らかにした。これらの結果は、EDsの臨界期曝露による行動神経内分泌学的変異を示すものであり、化学物質の遅発型影響の機序解明へ必要な知見を示している。また、早期指標を目指した母子分離誘発蹄鳴反応試験において抗不安薬の投与により性差が検出されることを示した。

A　研究目的

多くの動物は雌雄2つの性をもち、有性生殖を行う。その結果、子孫は多様性に富み、変化し続ける周辺環境へ柔軟な対応をすることが可能になる。この有性生殖を行うためには、雌雄が互いに性特異的な行動を示すことが必要不可欠となる。雌特異的あるいは雄特異的な行動は、脳により制御されることから脳にもまた「性」が存在する。性的に未分化な状態の脳は、卵巣や精巣から分泌される性ホルモンにより性分化の方向性が決定される時期が存在し、これを臨界期と呼ぶ。ラットの臨界期は、出生前5日から出生後5日の間に存在すると報告されており、この時期に性ホルモン様作用をもつ化学物質の曝露を受けると正常な脳の性分化が阻害される場合がある（Negri-Cesi et.al., J Steroid Biochem Mol Biol. 2008; Golub et al., Birth Defects Res B Dev Reprod Toxicol. 2010）。昨年度まで、私たちはエストロゲン様物質（EDs）の1つである17α-etynyl estradiol（EE）を生後24時間以内（周生期）に単回曝露し、その成長後の社会性行動などへの影響を調査した。その結果、EE曝露は濃度依存的に雌ラットの性選好性を消失させること、性行動中の雌特異的な行動を抑制することを見出した。これらの結果は、周生期の高濃度エストロゲン様物質曝露によって、正常な脳の雌化が阻害され、成熟後の雌特異的な行動の一部が抑制される可能性を示唆している。

室内ダスト中に検出される難燃剤の1つであるTriphenyl phosphate（TPhP）は、培養細胞を用いた（in vitro）研究からエストロゲン作用を持つ化学物質であると報告されている（Suzuki et al., Environ Sci. Technol. 2013）。しかしながら、TPhPが動物の体内（in vivo）に取り込まれた場合、エストロゲン様物質としてどのような影響をもつか、さらには、幼若期あるいは成熟期といった異なる成長段階にある動物へどのような影響をもつかもまた不明である。これまでに、周生期雄ラットに対し、TPhPを28日間連続で経口投与し成長後の社会性行動を解析した。その結果、TPhPを連続曝露された雄ラットは、雌ラットとの性行動中の雄特異的な行動の一部が低下することを見出した。

そこで本年度は、周生期雌ラットに対し、EE および TPhP を 28 日間経口投与し、成長後の社会性行動を解析することで、雌ラットへの EE ならびに TPhP の影響評価を行うことを目的とした。このため、本研究では以下の 2 つの解析を行った。

（1）発達への影響を評価するために、連続経口投与終了直後である 4 週齢（幼若期、①）と 15 週齢（成熟期、⑤）の試験雌ラットの臓器重量を測定

（2）社会性行動への影響を評価するために、12−14 週齢試験雌ラットの性選好性試験（②）、試験雌ラットと同週齢の雄ラット（③）あるいは同週齢の雌ラット（④）との性行動を解析

上記の実験に加えて、ED s の行動学的早期指標を確立するために、母子分離誘発啼鳴反応試験にて性差が検出される条件について検討した。

B 研究方法

＜EE ならびに TPhP 周生期曝露の影響＞

生後 24 時間以内の Wistar-Imamichi 系雌ラットに対して、Sesame oil、Sesame oil に溶解した TPhP（和光純薬工業株式会社）、あるいは EE（Sigma Aldrich, USA）を 28 日間連続で経口投与した。それぞれの物質の 1 日あたり投与量は、Sesame oil は 5 ml/kg（X-Ctrl 群）、TPhP は 25 mg/kg（X-LTP 群）あるいは 250 mg/kg（X-HTP 群）、EE は 15 μg/kg（X-EE 群）とし、4 群を作出した。また、周生期雄ラットに対して Sesame oi（5 mg/kg/day）を 28 日間連続で経口投与した群（Y-Ctrl 群）を同時に作出した。これらの雌雄ラットは一腹あたり 8 匹ずつになるよう里子操作を行い、生後 21 日齢で離乳した。離乳した雌雄ラットは各ケージ 4 匹ずつ、同性で群飼育した。

①幼若期臓器重量測定

4 週齢時、各試験雌ラット群のうち半数の体重を測定し、剖検後、肝臓、腎臓、卵巣、そして子宮の各臓器の重量を測定した。また同時に生殖器から肛門までの距離（Anogenital distance）を測定した。

各群、残り半数は 8 週齢時に卵巣摘出術（OVX）を行い、卵巣由来の性ホルモンによる影響を排除した。これらの OVX 雌ラットを行動試験に用いる場合は、試験開始 48 時間前に Estragiol benzoate（EB）を 0.5 μg/ 0.1 ml、さらに 4 時間前に Progesterone を 500 μg/ 0.1 ml、それぞれ皮下投与し、発情誘起処置を行った。

②性選好性試験

黒色塩化ビニル製ケージへ成熟雌ラットおよび雄ラット床敷を左右それぞれへ設置した。その後、試験開始前に発情誘起処置を行った 12 週齢の各群雌ラットおよび雄ラットを単独で導入し、5 分間の行動を上部から撮影し録画した。これらの動画を解析し、雌雄床敷領域への接近行動時間・滞在時間を測定した。

③雄ラットとの性行動試験

性選好性試験終了後、雌ラットは透明アクリル製ケージへ移動し、60 分の馴化後、同週齢の性経験済み相手雄ラットを導入し、60 分間の行動を正面から撮影し録画した。これらの動画を解析し、相手雄ラットのマウント行動発現回数、試験雌ラットの誘惑行動（Ear wiggling、Hopping）、拒否行動、そして雄受容姿勢（Lordosis）の発現回数を測定した。さらに、雄受容姿勢発現回数は、雄ラットのマウント行動発現回数に対する割合を示す Lordosis 商に用いた。

④雌ラットとの性行動試験

試験③終了から 1 週間後、再び試験雌ラットへ発情誘起処置を行い、試験③と同様の透明アクリル製ケージへ移動し、60 分の順化後、同週齢の発情誘起処置済み OVX 相手雌ラットを導入し、60 分間の行動を正面から撮影し録画した。これらの動画を解析し、試験雌ラットのマウント行動発現回数、誘惑行動（Ear wiggling、Hopping）、攻撃行動を測定した。

⑤成熟期臓器重量測定

OVX の際に体重と卵巣重量を測定した。また全ての行動試験終了後、各群の試験雌ラット（15 週齢）の体重を測定し、剖検後、肝臓、腎臓、そして子宮の各臓器重量を測定した。また同時に Anogenital distance を測定した。

＜母子分離誘発啼鳴反応の性差＞

0 週齢 Wistar-Imamichi 系雌ラットにおいて、母親および同腹仔から引き離されると発する、20 kHz～60 kHz の超音波領域に主成分を持つ啼鳴反応を指標に、啼鳴反応における 3 時間

前のジアゼパム経口投与の影響が、性差を有するか否か検討した。仔ラットを母獣から分離後、すぐに防音箱内のシャーレに入れ 5 分間測定した。鳴き声はマイクロホン（CO-100K、三研マイクロホン株式会社）で集音してアンプ（OCTA-CAPTURE、ローランド株式会社）で増幅し、Spectra PLUS 5.0 (Pioneer Hill Software、Poulsbo)にて解析した。

＜倫理面への配慮＞

動物の管理、行動試験の実施は明治大学農学部動物実験委員会の許可の下で行った。特に、行動試験は、動物に対し耐え難い苦痛を与えないよう十分な配慮のもと行った。

C　研究結果

＜EE ならびに TPhP 周生期曝露の影響＞

①幼若期臓器重量測定

測定した体重について群間で差はなく、各化学物質の影響は認められなかった（$F_{(3, 10)} = 2.05, P = 0.11$）（表 1）。また、体重あたりに換算した各臓器重量について群間で差は認められず、各化学物質の影響は認められなかった（肝臓：$F_{(3, 10)} = 0.87, P = 0.52$、腎臓：$F_{(3, 10)} = 1.39, P = 0.27$、卵巣：$F_{(3, 10)} = 0.37, P = 0.86$、子宮：$F_{(3, 10)} = 1.61, P = 0.20$）（表 1）。Anogenital distance については、群間で差が認められ（$F_{(4, 61)} = 27.22, P < 0.01$）、Y-Ctrl 群がその他全ての群と比較して長くなったが（$P < 0.01$）、各化学物質の影響は認められなかった（図 1A）。

②性選好性試験

性選好性試験では、各群について雌床敷への接近行動時間と雄床敷への接近行動時間の差（性選好性スコア）について比較したところ、群間に差が認められ（$F_{(4, 35)} = 4.61, P < 0.01$）、X-EE 群と Y-Ctrl 群の性選好性スコアが、X-Ctrl 群のそれと比較して高いことが明らかになった（$P < 0.05$）（図 2）。一方で、X-LTP 群あるいは X-HTP 群の性選好性スコアは、いずれの群とも差がなかった（図 2）。

③雄ラットとの性行動試験

性行動試験では、各群に対する相手雄ラットのマウント行動発現回数に群間で差が認められ（$F_{(3, 27)} = 3.87, P < 0.05$）、X-EE 群へのマウント行動発現回数が、X-LTP 群と比較して少ないことが明らかになった（$P < 0.01$）（図 3A）。また、試験雌ラットの Lordosis 商に群間で差が認められ（$F_{(3, 27)} = 18.34, P < 0.01$）、X-EE 群の Lordosis 商が、他の全ての群と比較して低いことが明らかになった（$P < 0.01$）（図 3B）。試験雌ラットの誘惑行動の 1 つである Ear wiggling の発現回数にも群間で差が認められ（$F_{(3, 27)} = 8.08, P < 0.01$）、X-EE 群の Ear wiggling 発現回数が、他の全ての群と比較して少ないことが明らかになった（$P < 0.05$）（図 3C）。さらに Hopping 発現回数に群間で差が認められ（$F_{(3, 27)} = 5.80, P < 0.01$）、X-HTP 群と X-EE 群の Hopping 発現回数が、X-Ctrl 群と比較して少ないことが明らかになった（$P < 0.01$）（図 3D）。一方で、X-LTP 群の Hopping 発現回数は、他のすべての群と比較して差は認められなかった（図 3D）。そして雄のマウント行動に対する拒否行動の発現回数は群間で差は認められなかった（$F_{(3, 27)} = 1.35, P = 0.28$）（図 3E）。

④雌ラットとの性行動試験

性行動試験では、雌ラットに対する各試験雌ラットマウント行動発現回数に群間の差は認められなかった（$F_{(3, 27)} = 4.07, P = 0.78$）（図 4A）。また、試験雌ラットの誘惑行動のいずれの発現回数にも群間の差は認められなかった（Ear wiggling: $F_{(3, 27)} = 1.34, P = 0.28$; Hopping: $F_{(3, 27)} = 0.91, P = 0.45$）（図 4C, D）。そして雌ラットに対する攻撃行動の発現回数も群間の差は認められなかった（$F_{(3, 27)} = 2.15, P = 0.12$）（図 4B）。

⑤成熟期臓器重量測定

OVX 時（8 週齢）に測定した試験雌ラットの体重に群間で差が認められ（$F_{(3, 60)} = 8.75, P < 0,01$）、X-EE 群の体重が他の全ての群と比較して増加していることが明らかになった（$P < 0.05$）（表 2）。また体重あたりに換算した卵巣重量について群間で差が認められ（$F_{(3, 60)} = 7.51, P < 0.01$）、X-EE 群の卵巣重量が低下していることが明らかになった（$P < 0.05$）（表 2）。

全ての行動試験終了後の 15 週齢時には、測定した体重について群間で差が認められたが（$F_{(5, 21)} = 3.99, P < 0.05$）、各化学物質の影響は認められなかった（表 3）。また、体重あたりに換算した各臓器重量については群間で差はなく、各化学物質の影響は認められなかった

（肝臓：$F_{(5, 22)} = 1.18, P = 0.36$、腎臓：$F_{(5, 22)} = 2.32, P = 0.08$、子宮：$F_{(5, 21)} = 1.92, P = 0.15$）（表3）。 Anogenital distance については、群間で差が認められ（$F_{(4, 46)} = 116.03, P < 0.01$）、Y-Ctrl群がその他全ての群と比較して長くなったが（$P < 0.01$）、各化学物質の影響は認められなかった（図1B）。

＜母子分離誘発啼鳴反応の性差＞

ジアゼパム 1 ml/kg 投与による母子分離誘発蹄鳴反応の低下が雄で有意に表れる一方、雌では表れにくいという性差を検出した。

D 考察

＜EE ならびに TPhP 周生期曝露の影響＞

エストロゲン様物質の発達への影響を評価するために、幼若期および成熟期のそれぞれの体重と化学物質の代謝に関わる肝臓、体外の排泄へ関わる腎臓、エストロゲンの主要産生組織である卵巣、そして性ホルモンの作用部位の 1 つである子宮、これら各臓器の重量を測定した。その結果、化学物質の曝露終了直後では、体重と各臓器重量へ化学物質の影響は認められないことが明らかになった。その一方で、性成熟完了直後の 8 週齢時では、EE 曝露によって体重の増加と卵巣重量の低下が認められた。成熟期では、ふたたび体重や卵巣以外の各臓器重量への化学物質の影響が認められなかった。これらの結果は、周生期 EE 曝露が、卵巣組織の発達を抑制していること、さらには体重の増加が認められたことから摂食行動を抑制する卵巣由来のエストロゲン分泌量の低下、あるいはエストロゲンの作用点であるエストロゲン受容体の活性低下を引き起こしている可能性が示唆された。一方で、個体の成長にともない顕著に雌雄で異なる長さを示す Anogenital distance については、幼若期および成熟期のいずれの時期において、化学物質の影響は認められなかった。

社会性行動への影響を評価するために、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。その結果、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、EE 曝露によりその性選好性が雄型化する可能性が示唆された。その一方で、TPhP 曝露は、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。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。その結果、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。一方で、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。成熟雌ラットとの性行動試験では、試験雌ラットのマウント行動、誘惑行動、攻撃行動のいずれの行動発現回数についても化学物質の影響は認められなかった。しかしながら、EE 曝露によって雌ラットへの攻撃行動が増加していることや、TPhP は曝露濃度の違いにより、雌ラットに対してマウント行動が増加する、あるいは攻撃行動が増加することが見出せつつある。これらの行動試験の結果は、周産期のエストロゲン様物質の連続曝露によって、成熟後の雌特異的な社会行動の一部が抑制、あるいは消失し、さらには雄特異的な社会行動の発現が起こることを示している。

以上の結果から、周産期雌ラットへのエストロゲン様物質曝露、特に EE 連続曝露は脳の正常な性分化に影響を与え、卵巣の発育あるいは機能を抑制し、成熟後の雌特異的な性行動を抑制する可能性が示唆される。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。

＜母子分離誘発啼鳴反応の性差＞

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

本年度は、雌ラットに対して、EE ならびに in vitro でエストロゲン様作用をもつ化学物質 TPhP を周生期に 28 日間連続で経口投与し、in vivo 条件下でどのような影響をもつかを検討した。その結果、幼若期の体重や臓器重量に影響がないが、EE 曝露は性成熟完了に至るに従い、エストロゲンの主要産生臓器である卵巣の発育を抑制することを示した。また、EE 曝露によって、成熟後の性行動中の雌特異的な行動が抑制されること、TPhP はその曝露濃度によって性行動中の誘惑行動の一部が抑制されることが明らかになった。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。

F 研究発表

1 論文発表

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2） 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. 2016 Jan 7;11(1):e0146136

2 学会発表

1）田邉郁也、小峰千亜希、吉田緑、川口真以子: 生後 24 時間以内の雌ラットへの ethynyl estradiol 曝露が受動回避学習に及ぼす影響: 第 62 回日本実験動物学会総会（2015 年 5 月 28 日～30 日、京都）

2）立川直之、志賀健臣、中村孝博、小峰千亜希、堀井康之、渡辺元、田谷一善、溝口康、吉田緑、川口真以子: 雌ラットへの生後 24 時間以内 ethynyl estradiol 曝露が大脳皮質と海馬の estrogen receptor(ER)α 及び ERβ 発現に及ぼす影響: 第 62 回日本実験動物学会総会（2015 年 5 月 28 日～30 日、京都）

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4）中山愛里、服部達哉、大河原利、田辺郁也、磯部安菜、宍戸浩孝、鈴木剛、滝上英孝、川口真以子: 幼若期雌ラットへの ethynyl estradiol と triphenyl phosphate の 28 日間曝露が成熟後の性行動に及ぼす影響: 第 42 回日本神経内分泌学会・第 23 回日本行動神経内分泌研究会合同学術集会（2015 年 9 月 17 日～19 日、宮城）

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6）磯部安奈、川口真以子: 仔ラットの母子分離誘発啼鳴反応の発声回数に対する抗不安薬ジアゼパムの抑制作用には性差がある: 第 89 回日本薬理学会年会（2016 年 3 月 9 日～11 日、神奈川）

G 知的財産権の出願・登録状況

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

表1　幼若期（4週齢）体重および臓器重量測定の結果

		Per kg			
Treatment	B.W.	Liver	Kidney	Ovary	Uterus
Ctrl	77.3 ± 5.98	43.0 ± 0.62	10.7 ± 0.32	0.3 ± 0.06	0.9 ± 0.27
LTP	81.5 ± 3.41	43.1 ± 1.68	9.8 ± 0.41	0.4 ± 0.04	0.9 ± 0.11
HTP	79.7 ± 1.59	49.8 ± 1.99	10.2 ± 0.21	0.4 ± 0.03	1.0 ± 0.13
EE	86.7 ± 3.34	46.9 ± 1.48	10.8 ± 0.40	0.3 ± 0.05	2.1 ± 0.22

表2　性成熟完了後（8週齢）体重および卵巣重量測定の結果

	Per kg	
Treatment	B.W.	Ovary
Ctrl	225.2 ± 3.49	0.7 ± 0.01
LTP	226.3 ± 3.06	0.6 ± 0.01
HTP	227.2 ± 4.05	0.6 ± 0.01
EE	248.5 ± 3.97 *	0.4 ± 0.00 *

表3　成熟期（15週齢）体重および臓器重量測定の結果

		Per kg		
Treatment	B.W.	Liver	Kidney	Uterus
Ctrl	383.3 ± 21.88	35.2 ± 1.69	5.3 ± 0.30	0.3 ± 0.03
LTP	360.9 ± 1.22	35.3 ± 2.60	6.1 ± 0.26	0.9 ± 0.31
HTP	409.6 ± 10.70	35.9 ± 0.39	5.0 ± 0.06	0.4 ± 0.02
EE	434.0 ± 21.10	37.1 ± 2.09	5.2 ± 0.20	2.1 ± 1.13

表中のCtrlはSesame oil（5 ml /kg /day）曝露群、LTPは低TPhP（25 mg /kg /day）曝露群、HTPは高TPhP（250 mg /kg /day）曝露群、EEはEE（15 μg /kg /day）曝露群を示す。*: $P < 0.05$

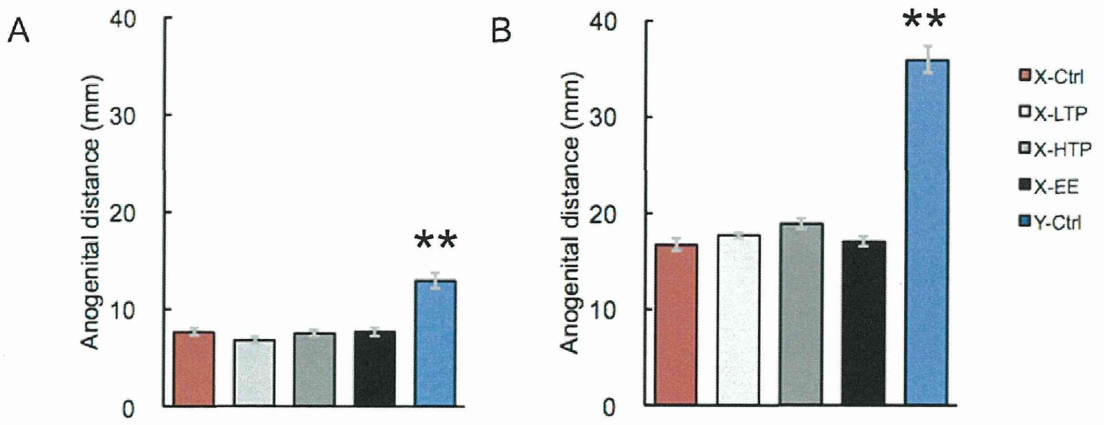

図1　幼若期（4週齢）および成熟期（15週齢）Anogenital distance測定の結果

幼若期のAnogenital distance測定の結果（A）、Y-Ctrl群がその他の全ての群と比較して長かった（P < 0.01）。成熟期のAnogenital distance測定の結果（B）も同様に、Y-Ctrl群がその他の群と比較して長かった（P < 0.01）。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 < 0.01

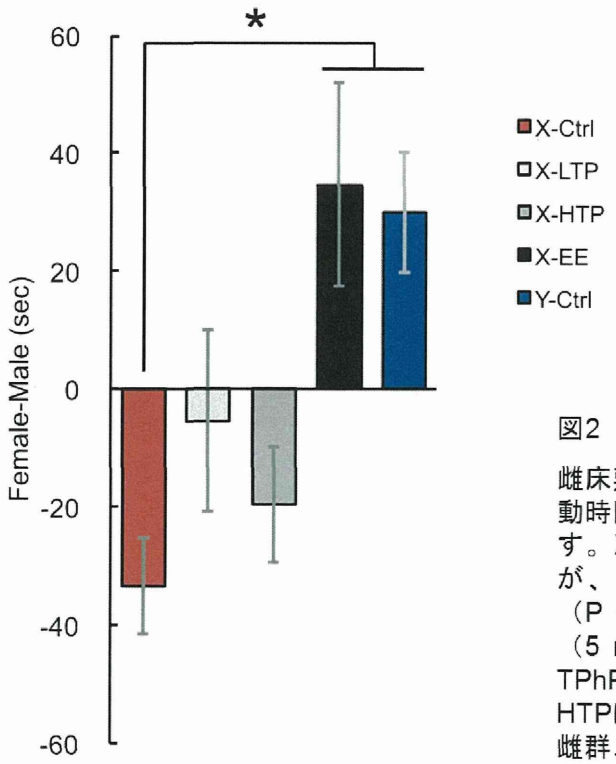

図2　12週齢時の性選好性試験の結果

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

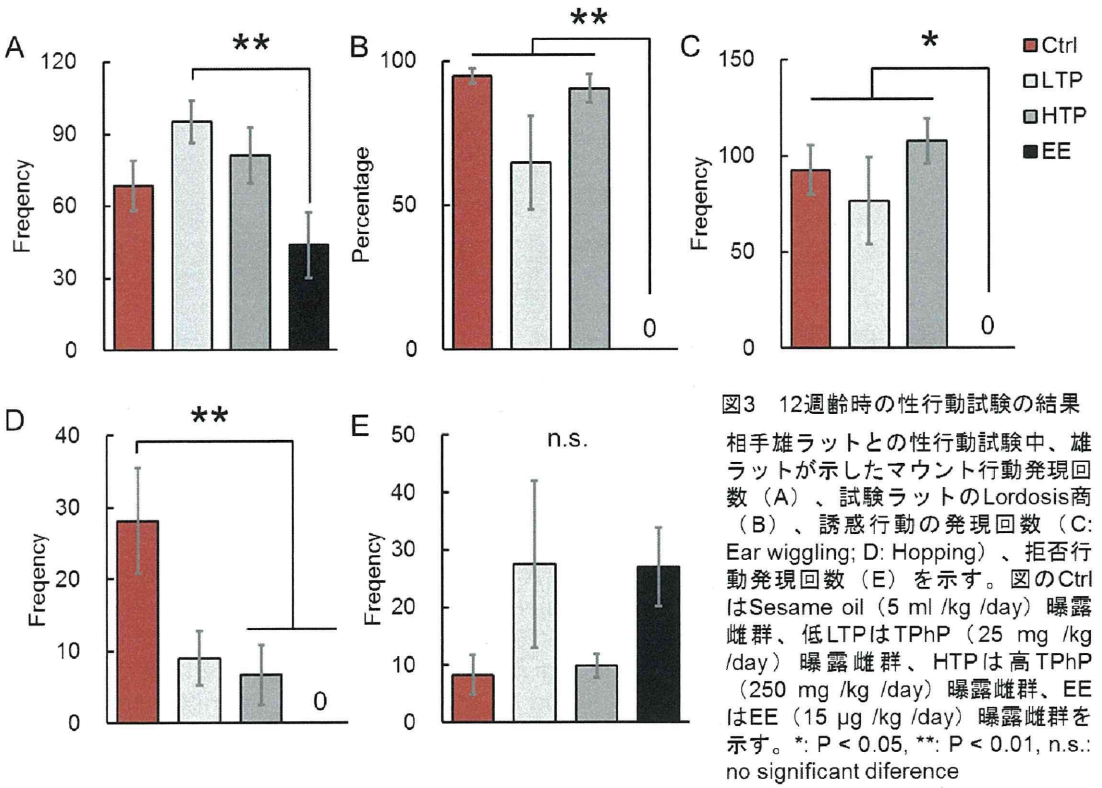

図3　12週齢時の性行動試験の結果

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, n.s.: no significant diference

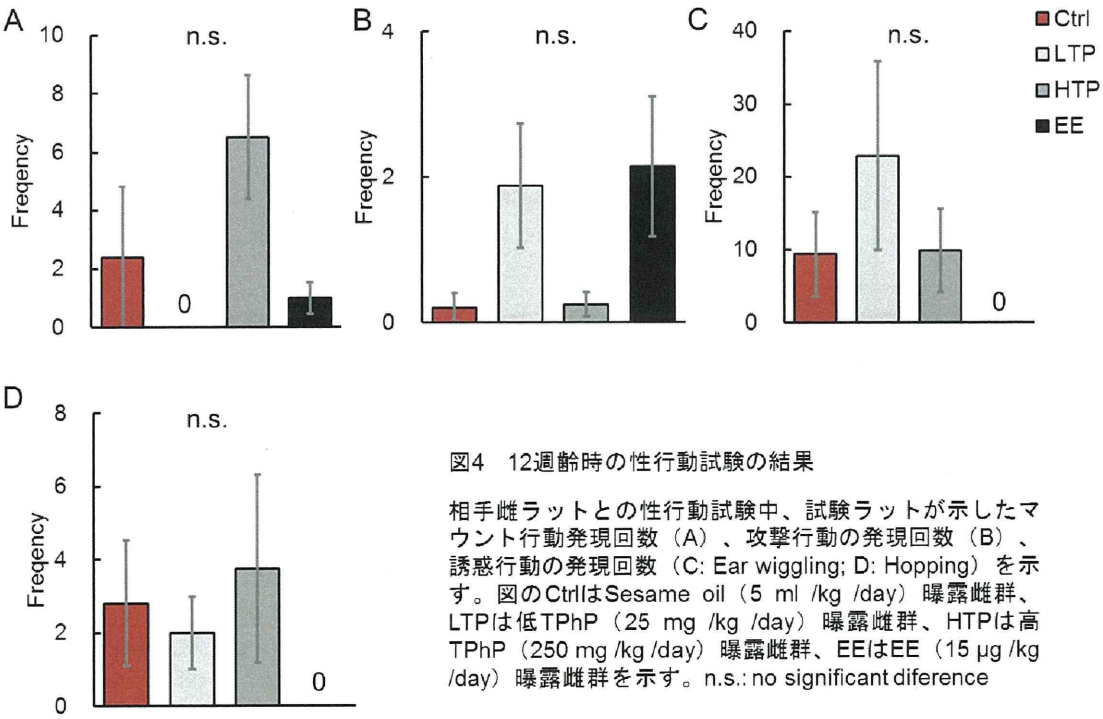

図4　12週齢時の性行動試験の結果

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	刊・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.	Reproductive Toxicology	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	Jan 7;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 (2), 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 Toxicology	51, 145-156	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.	Reproductive Toxicology	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.	Journal of Applied Toxicology	Online Jul 14	2015

Ⅳ. 研究成果の刊行物

Reproductive Toxicology 60 (2016) 33–38

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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

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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).

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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

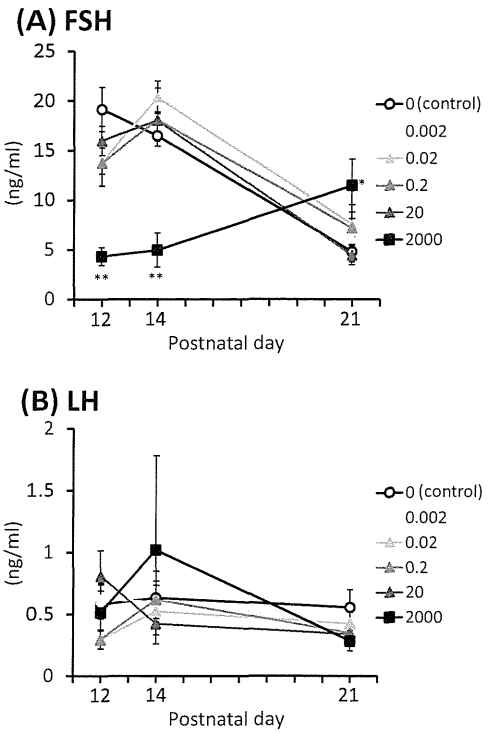

Fig. 1. Serum FSH (A) and LH (B) levels at postnatal day 12, 14 and 21 in female rats that received a single injection of EE. Data represent the mean ± SEM. $n = 5$ per group. *, **: Significantly different from the 0 μg/kg group at $p < 0.05$ and 0.01, respectively (Dunnet's test).

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

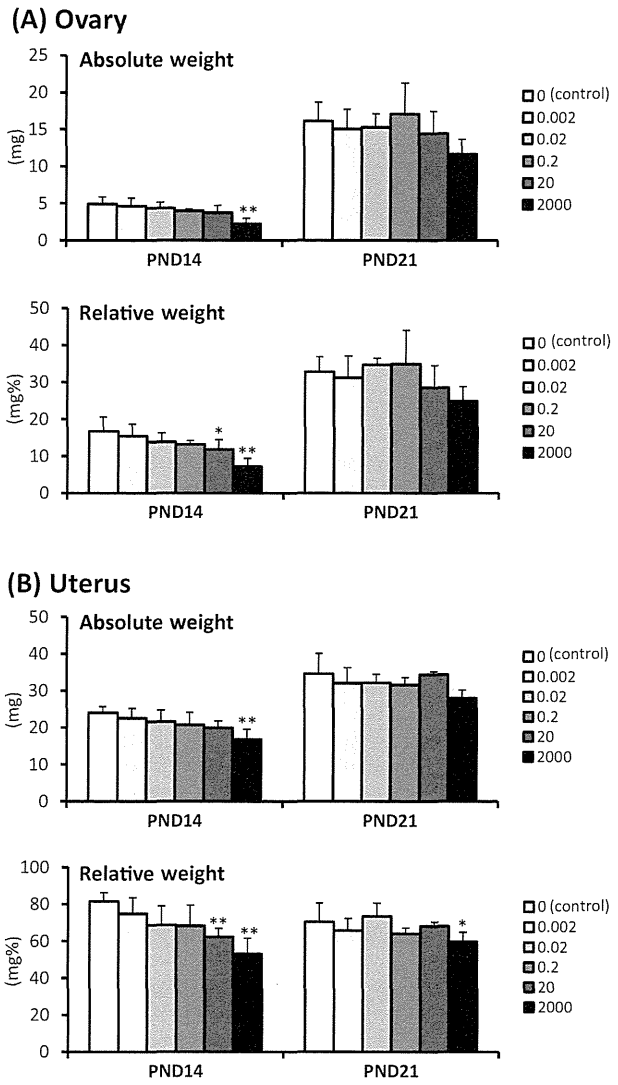

Fig. 2. Weights of the ovaries (A) and uterus (B) at postnatal day 14 and 21 in female rats that received a single injection of EE. Data represent the mean ± SD. $n = 5$ per group. *, **, Significantly different from the 0 μg/kg group at $p < 0.05$ and 0.01, respectively (Dunnet's test).

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.

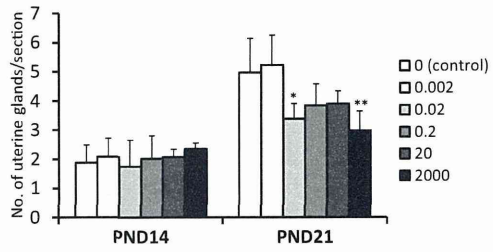

Fig. 3. Number of uterine glands per section at postnatal day 14 and 21. Data represent the mean ± SD. $n = 5$ per group. *, **, Significantly different from the 0 μg/kg group at $p < 0.05$ and 0.01, respectively (Dunnet's test).

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

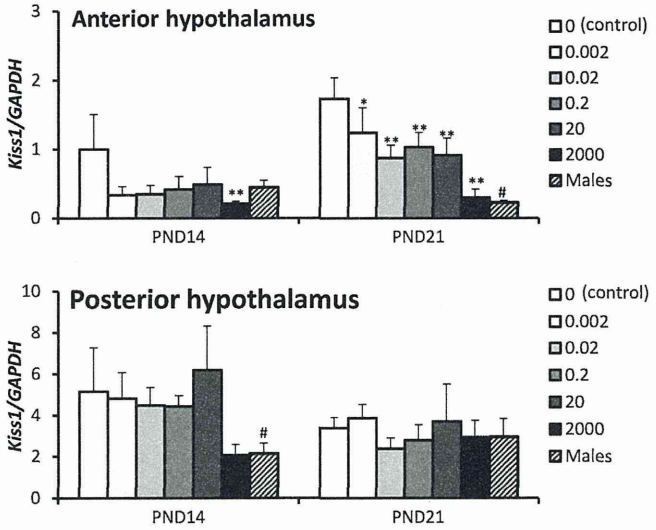

Fig. 5. Kiss1 expression in the anterior and posterior hypothalamus at postnatal day 14 and 21. Data represent the mean ± SD. $n = 5$ per group. *, **, Significantly different from the 0 μg/kg group at $p < 0.05$ and 0.01, respectively (Dunnet's test). #: $p < 0.05$ vs. 0 μg/kg group (Student's t-test).

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

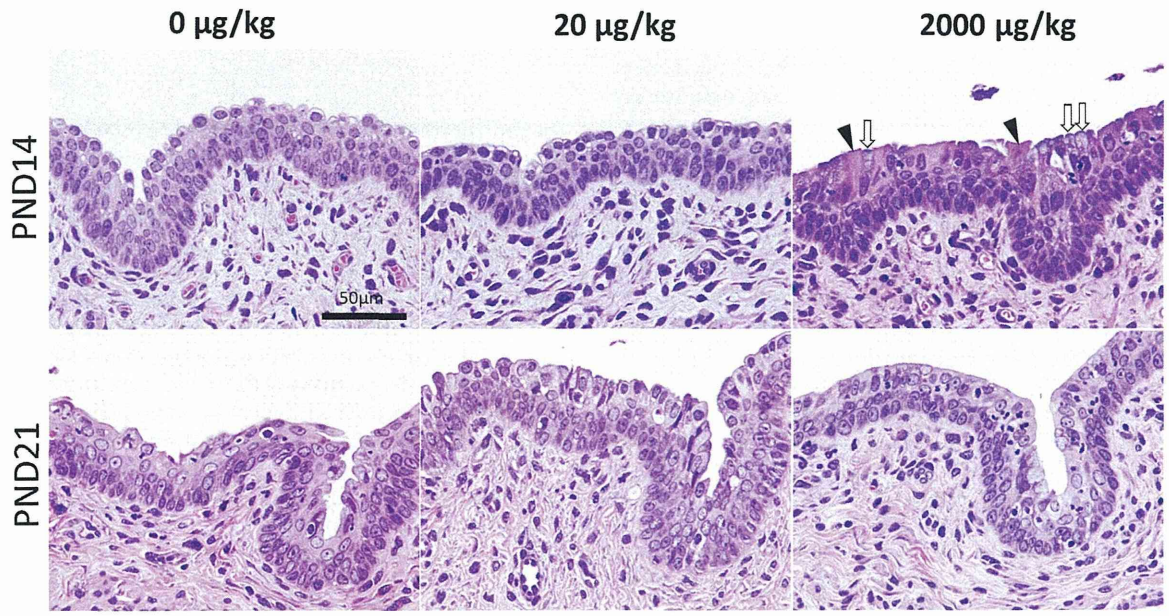

Fig. 4. Histology of the vaginal mucosa at postnatal day 14 and 21. Eosinophilic keratinized cells (black arrowhead) and mucinous cells (white arrow) were observed in the vaginal mucosa only in the 2000 μg/kg group at PND14. HE stain. Scale bar: 50 μm.

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

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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

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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

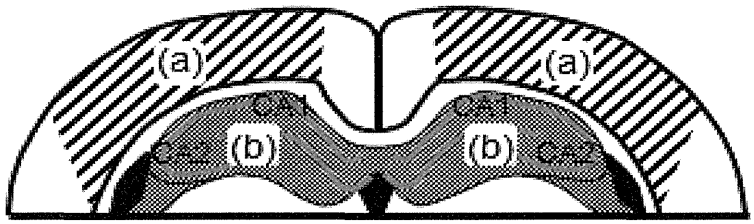

Fig 1. Illustration of a coronal section of the brain showing tissue sampling sites for the cortex (a) and hippocampus (b) [25]. CA1: CA1 region of the hippocampus; CA2: CA2 region of the hippocampus.

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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 $P < 0.05$.