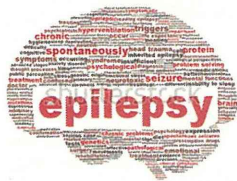
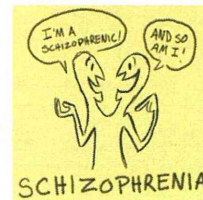


GFAP level and glial density: Implications in psychiatric diseases



- 'Adult onset' psychiatric diseases
VS
diseases of neurodevelopment

- Developmental exposure of BPA which led to reduced GFAP level and density, together with corpus callosum hypertrophy ----- new animal disease model that reflects gene-environment interactions and developmental processes.



Acknowledgement



Nara Institute of Science and Technology (NAIST)



Nakashima LAB

Nat Inst Health Sci

Tohoku Univ.

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研究要旨 胎生期から離乳期にかけた母体経由のビスフェノールA暴露が、仔の乳幼児期および成長後の認知・情動行動に与える影響について、平成23年度に行った包括的行動解析の研究結果をもとに、主として情動性の変化について新規行動実験系を立ち上げて詳細な解析を試みた。その結果、ビスフェノールA暴露群において、乳児期における衝動性の有意な低下、および成長期以降の成体における環境刺激に対する反応性の鈍化傾向が示唆された。

A. 研究目的

胎児期・乳児期に母体経由で暴露されたビスフェノールAの影響については、様々な研究が行われてきたが、必ずしも一貫した結果が得られているわけではない。特に行動・精神機能に及ぼす影響については小児期から成熟期を対象とした断片的な知見が得られているに過ぎず、生涯発達の視野における研究は皆無と言える。そこで本研究では、マウスをモデル動物とした新規行動解析実験系を構築し、発生-発達期におけるビスフェノールA暴露の行動発達に対する影響について横断的および縦断的な解析を可能とする方法論を確立することを目的としている。

平成23年度の研究においては、遺伝子改変マウスに用いられている汎用の包括的行動テストバッテリーを適用することによって、発生・発達期における母体経由のビスフェノールA暴露に起因すると考えられる重要な行動変化を見いだすことができた。しかし同時に、包括的行動テストバッテリーのようなマクロ的視野における解析法では、環境汚染物質や低濃度の化学物質による行動・精神機能に対する影響を検討するには解析精度に限界があることが明らかになった。

そこで本年度は、平成23年度の研究から明らかになった胎児期・乳児期に母体経由で暴露されたビスフェノールAによる情動機能の変化について精度の高い新規行動課題を構築し、より詳細な検討を行うことを目的とした。

B. 研究方法

被験体・処置：C57BL/6J系統のマウス（日本クレアおよび日本SLC：8週齢）を購入後、1週間の馴致期間において、交配を行った（10日間）。交配はマウスをランダムに2群に分け、一方には飲用水中にビスフェノールA（以下BPA：1ppm）、他方には溶媒として0.01%エタノール（以下Veh）を添加した。BPAおよびVehの添加は離乳時（4週齢）まで行った。妊娠確認後は毎朝妊娠マウスの状態を確認し、出産が認められた日を生後0日（PD 0）とした。

乳幼児期における発達検査：平成23年度と同様にPD 5から身体的発達および乳児反射について観察・評価を行った。

乳幼児期における情動性評価：平成23年度に見られた恐怖条件付けの変化と比較し得る強度の高い情動反応として、新奇物体に対する衝動的逃避反応に焦点をあてて評価を行った。生後18日齢のマウスを中央に新奇なフギュア（直径3cm、高さ5cm）を配置したオープンフィールド（20 x 20 x 20 cm）に入れ、マウスの行動を3分間観察した。通常のオープンフィールド行動の指標（移動距離、不動時間）と同時に、フィギュアへの接触反応とフィギュアおよびオープンフィールドからの衝動的逃避反応（壁面に対するジャンピング反応）の回数を計測した。

成体における情動性評価：平成 23 年度の研究においては 11-20 週近傍の比較的若い時期の成体における行動変化について検討したことから、平成 24 年度は 35 週齢以降の成体について検討を行った。行動テスト経験の影響を除外するために、マウスは実験予定週齢まで通常の飼育管理作業の操作以外は一切行わずに飼育した。成体を用いた実験においては、後述する上記乳児期実験の結果を踏まえて、抑制的情動性の変化について検討するための課題として、自発的環境選好試験とトレッドミルにおける強制走行試験を採用した。自発的環境選好試験では、オープンフィールドの床面の半分に平坦なアクリル板と、残りの半分に同素材のアクリル板に凹凸を施したものを敷き、その上にマウスを放置して各々の領域における滞在時間を測定した。また、トレッドミル強制走行試験では、マウスをトレッドミル(幅 5 cm、長さ 20 cm、秒速 15 cm)の走路内に入れ、20 秒間マウスを強制走行させ、走行を諦めた個体数および走行を諦めるまでの時間を測定した。

感覚受容器に関する組織学的検討：末梢の感覚受容器の変化を調べるために、実験後マウスを還流固定し左足肉球部(footpad)の凍結切片を作成し、組織染色を行った。

(倫理面への配慮)

動物実験における 3R の原則に従い、使用動物数の削減に努めた。なお、本研究における動物実験は、独立行政法人理化学研究所実験動物委員会の承認を得て、同動物実験倫理規定を遵守して行われた。

C. 研究結果

妊娠・出産への影響：本研究では、合計 10 ペアの交配に成功した(乳幼児実験：8 ペア、成体実験：2 ペア)。年度途中で親マウスの飼育業者が変更になったが、出産・育児行動に差異は認められなかった。出産数および子の性比について χ^2 乗検定を行ったところ、いずれについても BPA 群と Veh 群の間に有意な差は認めら

れなかったが、BPA 群の妊娠個体数が Veh 群よりも多かった(BPA: 6, Veh: 4)

乳幼児期における発達検査：身体的発達については、平成 23 年度同様、PD5 および PD18 時点において BPA 群で体重が有意に高い傾向が見られた(drug: $F(1, 77)=36.8$, $p<0.001$; day: $F(1, 77)=1341.7$, $p<0.001$; drug x day: $F(1, 77)=9.42$, $p<0.005$, Two-way ANOVA)。一方、各乳児反射については有意な差は認められなかった。

乳幼児期における情動性評価：オープンフィールドの中央部にフィギュアを配置し、マウスをオープンフィールドの 4 角のいずれかに置いて実験を開始した。3 分間の測定の結果、移動距離および不動時間については BPA 群と Veh 群の間に有意な差は見られなかった。同様にフィギュアに対する接触行動の各指標(接触潜時、接

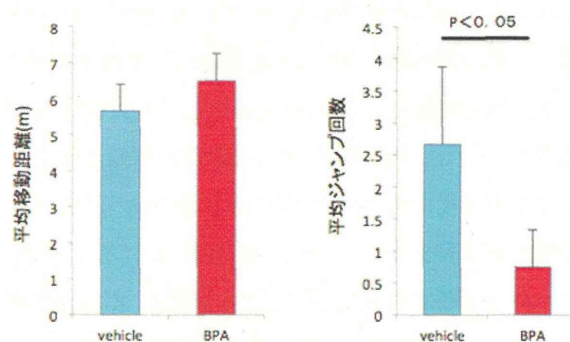


図1 幼児情動性試験(新奇物体テスト)の結果

触回数、接触時間)にも有意な差は認められなかった。一方、フィギュアあるいはオープンフィールドからの強い逃避行動(ジャンピング)の回数は BPA 群において有意に減少していた($U=122$, $p<0.05$ Fig. 1)。

成体における情動性評価：自発的環境選好試験において、オープンフィールド指標(移動距離、不動時間)については両群に差が認められなかったが、凹凸領域での滞在時間については BPA 群のマウスにおいて有意な伸張が認められた($U=0$, $p<0.05$: Fig. 2)。一方、トレッドミルによる強制走行試験においては、両群ともに走行を諦める個体は見られなかった。

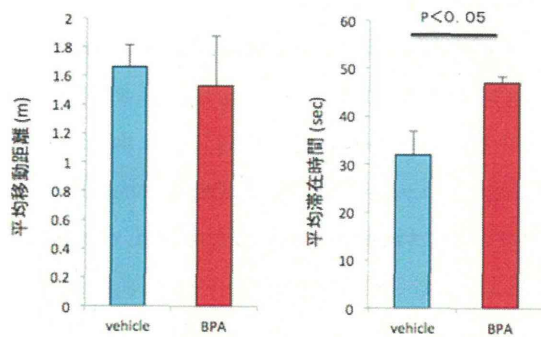


図2 自発的場所選好性試験の結果

感覚受容器に関する組織学的検討：自発的環境選好試験の結果、BPA 群のマウスがオープンフィールドの凹凸床面領域を選好したので、末梢の感覚受容器の変化について検討した。実験後マウスを還流固定し左足肉球部(footpad)の凍結切片を作成し、ヘマトキシリン・エオジン染色を行ったが、BPA 群と Veh 群の間に形態的变化は認められなかった。

D. 考察

本研究では、平成 23 年度の研究から明らかになった胎児期・乳児期に母体経由で暴露されたビスフェノール A による情動機能の変化について、汎用の行動解析課題よりも精度の高い新規行動課題を構築し、より詳細な検討を行うことを目的とした。平成 23 年度の研究において音と環境を手がかりとした恐怖条件付けにおいて BPA 群が有意に高い恐怖反応を示したことから、本年度は情動機能の中でも特に衝動性と感覚の鋭敏化に焦点を絞った実験を行った。

本研究では乳幼児期における情動性評価法として、オープンフィールド内における新奇物体との対面場面を設定し、その際にマウスが示す突発的な逃避反応(ジャンピング)の測定を用いている。近年行動の認知的制御の観点から衝動性の研究が盛んになっているが、多くの研究では 5 肢選択課題(five-choice test)が用いられているが¹⁾、この課題は訓練が非常に困難である。これに対して、オープンフィールド内において新奇物体と対面させる方法は特別な訓練が不要で、マウスに対する持続的なストレス負荷も少ないため、乳幼児に

についても適用が可能な方法と言える。従来は新奇物体への接触等、被験動物の新奇物体に対する積極的な反応が指標として用いられてきたが²⁾、逃避のような消極的な反応を指標に加えることで、衝動性の変化についてより精度の高い測定が可能になると言える。ジャンピングは目視・自動計測どちらの場合でも比較的数値化が簡易なため、新奇物体への接触と同様、衝動性の測定指標として有効であると考えられる。また、本研究では、成体時における情動性変化の測定法として、自発的環境選好試験を用いた。この試験法は簡便ではあるがマウスの好悪に関連する情動の検出精度が高く³⁾、本研究においても BPA 群において感覚刺激に対する反応変化を明らかにしている。特別な装置を必要としないので、自由行動下の電気生理学的測定と組み合わせることでさらに有益な知見が得られるものと考えている。

本研究では、平成 23 年度の研究結果をもとに、BPA 暴露マウスが高強度の刺激(電気ショックや新奇物体)に対する反応性の亢進とともに、低強度の刺激に対する反応性の低下を示すことを明らかにした。しかし、このような BPA 暴露マウスにおける認知-情動性変化がどのような神経メカニズムによって生じているかは未だ不明である。そこで現在、伝統的なオペラント条件付けやパプロフ型条件付けの手法に電気生理学的な手法を合わせた実験系を構築中である。本年度の研究には間に合わなかったが、近い将来 BPA 暴露マウスにおける認知-情動性変化の生理学的メカニズムについて成果をあげられるものと考えている。

E. 結論

胎生期および授乳期において母体経由の BPA 暴露を受けた場合、その仔の身体的発達および成長後の情動反応に長期的な変化が認められた。これらの変化がどのような神経メカニズム変化によるものか確認するために、生理学的な検討が必要である。また、長期的な変化について、ヒトにおける更なる疫学的な研究の発展が望まれる。

F. 健康危険情報

特になし

G. 研究発表

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H. 知的財産所有権の出願・登録状況 (予定も含む)

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

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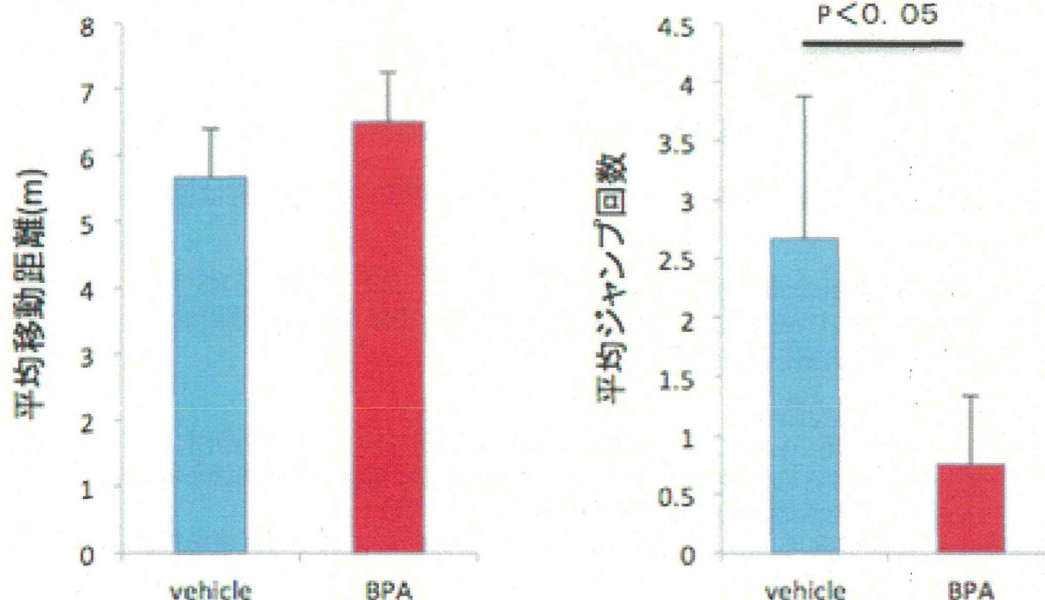


Fig. 1 Results of the open field test and assessment of impulsiveness



平成24年度厚生労働科学研究費補助金(化学物質リスク研究事業)
神経系発生-発達期の化学物質暴露による遅発中枢影響解析に基づく
統合的な情動認知行動毒性評価系確立に資する研究(H23-化学-一般-004)

発生-発達期ビスフェノールA 暴露の 行 動発達に対する影響解析

独立行政法人理化学研究所
脳科学総合研究センター・研究員

山田一之



1. これまでの経過と本年度の目的

胎児期・乳児期に母体経由で暴露されたビスフェノールAの影響については様々な研究が行われてきた。

△行動-精神機能に及ぼす影響について、生涯発達の視野における研究は僅少

本研究の目的:

マウスをモデル動物とした新規行動解析実験系を構築し、発生-発達期におけるビスフェノールA暴露の行動発達に対する影響について、横断的および縦断的な解析を可能とする方法論を確立すること

平成23年度の研究の経緯:

遺伝子改変マウスに用いられている汎用の包括的行動テストバッテリーの適用

○発生・発達期における母体経由のビスフェノールA暴露に起因すると考えられる行動変化を検出(自発活動性の変化、情動記憶の変化 等)

△環境汚染物質や低濃度の化学物質による行動-精神機能に対する影響を検討するには解析精度に限界がある

本年度の目的:

平成23年度の研究から明らかになった胎児期・乳児期に母体経由で暴露されたビスフェノールAによる情動機能の変化についてより精度の高い新規行動課題を構築し、さらに詳細な検討を行うこと

☆研究の動向

本年度は他グループから関連する重要な知見が報告されている

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Gestational and lactational exposure to bisphenol-A affects anxiety- and depression-like behaviors in mice.

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○胎生期、授乳期のBPA暴露が成長後の不安や鬱状態を亢進する

→平成23年度の山田の研究と一致した結果

[Neurotoxicology.](#) 2012 Oct;33(5):1390-5. doi: 10.1016/j.neuro.2012.09.002. Epub 2012 Sep 12.

A single exposure to bisphenol A alters the levels of important neuroproteins in adult male and female mice.

[Viberg H](#), [Lee J](#).

○新生児期のBPA暴露は単回暴露でも脳の発達に影響

→北嶋班における各研究と類似した結果

[Prog Neuropsychopharmacol Biol Psychiatry.](#) 2012 Dec 3;39(2):273-9. doi: 10.1016/j.pnpbp.2012.06.016. Epub 2012 Jul 1.

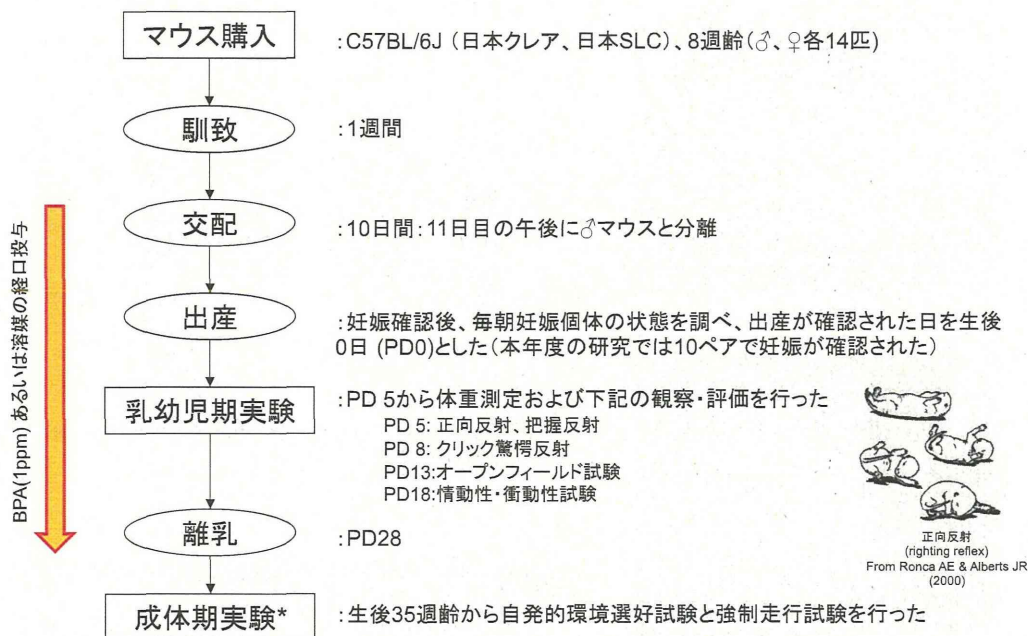
Effects of perinatal exposure to low dose of bisphenol A on anxiety like behavior and dopamine metabolites in brain.

[Matsuda S](#), [Matsuzawa D](#), [Ishii D](#), [Tomizawa H](#), [Sutoh C](#), [Nakazawa K](#), [Amano K](#), [Sajiki J](#), [Shimizu E](#).

○周産期におけるBPAの低用量暴露によって不安が亢進、またDAの代謝が低下

→低用量効果の神経シグナル伝達基盤に迫る？

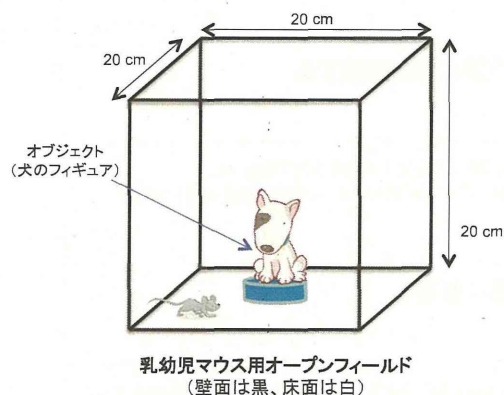
2. 方法



*乳幼児期の経験バイアスを避けるため、成体期実験は乳幼児期実験と異なる個体を用いた。成体期実験に用いたマウスは通常の飼育管理以外のハンドリングは一切行わなかった。

1) 幼児期における情動性評価

平成23年度に見られた恐怖条件付けの変化と比較し得る強度の高い情動反応として、新奇物体に対する衝動的逃避反応に焦点を当てた。



方法:

・マウスを中央に新奇なフィギュア(直径3 cm, 高さ5 cm)を配置したオープンフィールド(20 x 20 x 20 cm)に入れ、マウスの行動を3分間観察

観察指標:

- ・通常のオープンフィールド行動の指標 (移動距離、不動時間)
- ・フィギュアへの接触反応とフィギュアおよびオープンフィールドからの衝動的逃避反応(壁面に対するジャンピング反応)の回数

分析:

- ・画像解析→ANY-maze (Stoelting)
- ・統計解析→Excel統計 (ver 6.0, エスミ)

☆結果

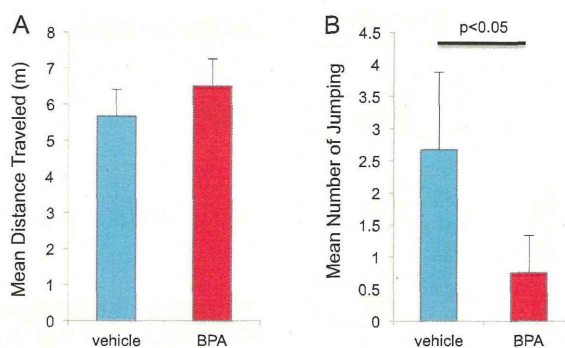
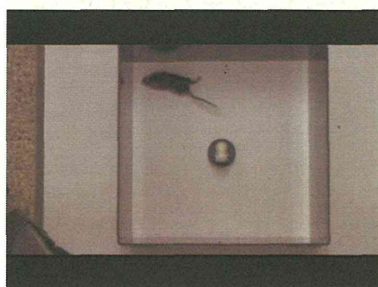
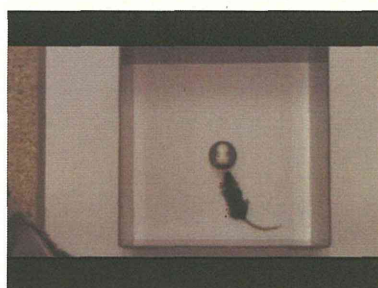
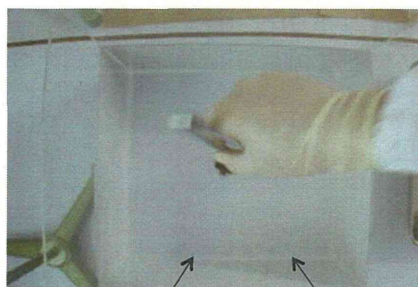


Fig. 1 Results of the emotional analysis in infants
A: mean distance of traveled (m), and B: mean number of jumping (impulsiveness)

胎生期・授乳期の母体経路のBPA暴露は、仔マウスの衝動性を低下させる可能性がある。
→「衝動性」と表現するとその低下は良い事のように思えるが、この場合好影響なのか悪影響なのか現時点では不明。

☆結果



アクリル板
(凹凸: 3 x 3 x 1 mm
の正四角すい模様)

アクリル板 (平坦)

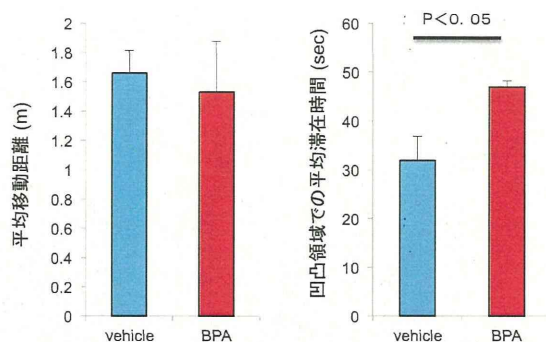


Fig. 2 Results of the spontaneous place preference test.



BPA暴露マウスでは、成体期において特定の床テクスチャに対する選好性が亢進している。
これは一体何を意味しているのだろうか？

2-2) トレッドミルにおける強制走行(歩行)試験



装置外観

方法:

- ・マウスを走路に入れる
- ・進行方向(ベルトの回ってくる方向)に向くのを待つ
- ・進行方向に向いたらベルト作動(15cm/sec)
- ・歩行反応を20秒間記録(100fps)
- ・inter trial intervalはおおよそ20~30秒
- ・試行数は5試行
- ・目視によって最も安定していると判断した試行について分析

観察指標:

- ・歩行パターン
- ・歩行/走行を諦めた回数

分析:

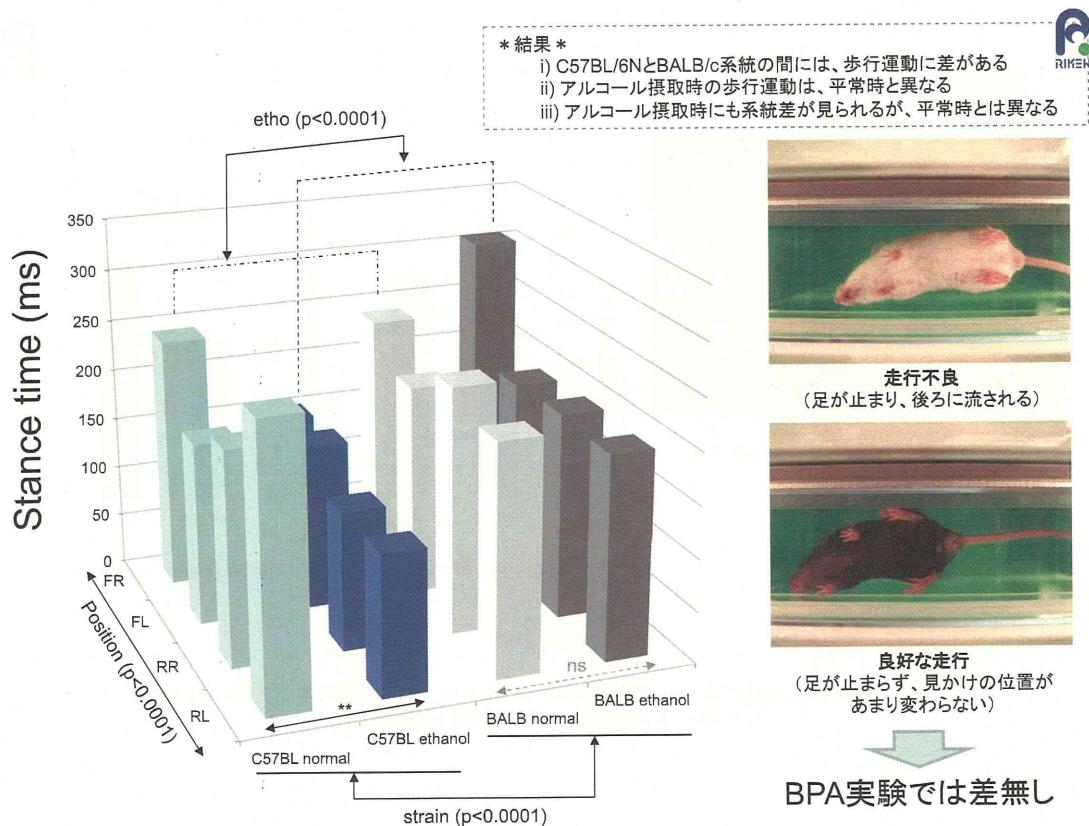
- ・画像解析→TreadScan (CleverSys)
- ・統計解析→Excel統計 (ver 6.0, エスミ)

☆予備実験: C57BL/6N, C3H/He, BALB/cによる再実験&アルコール投与実験

→C57BL/6とBALB/cの間に歩行運動の系統差があることを確認した
(C3H/Heについては確証を得られなかった)

→アルコール投与時の歩行運動について2系統で比較を行った

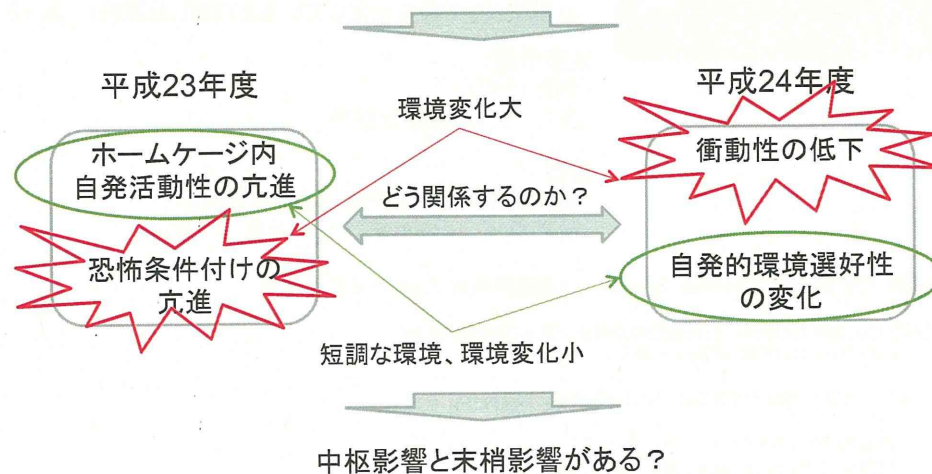
- ・投与量: 20%エタノール 10ml/kg(i.p.)
- ・投与後5分間home cageに置いてからテスト



3. 考察

本研究において、

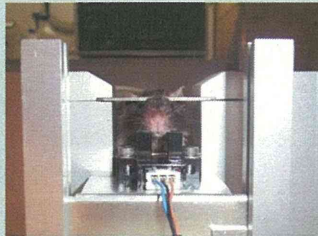
- 1) 胎児期・乳幼児期に母体経由でBPA暴露を受けたマウスは、幼児期の衝動性に変化が生じる可能性が示唆された。
- 2) BPA暴露群は、成体期において環境刺激(床テクスチャ)に対する選好性に変化を生じる可能性が示唆された。



中枢影響と末梢影響を分けて検討する必要があるかもしれない

中枢影響

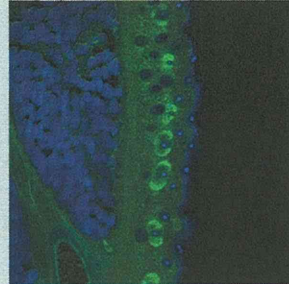
電気生理学的検討



頭蓋骨を露出させたマウスをヘッドプレートで実験装置に固定して、オペラント行動中の神経活動を測定する。装置と実験プログラムを開発中。

末梢影響

免疫組織学的検討



末梢(足裏)の感覚受容体の数や分布を調べる。ここではCK-20で染色しているが、上手くいかないなのでPGP 9.5 (UCH-L1)による染色を企画中。



5. 結論

○本年度の研究においても平成23年度の研究と同様に、胎生期および授乳期に母体がBPA暴露を受けた場合、その仔の身体的発達および成長後の行動に変化が見られた。

○胎生期および授乳期の母体経由のBPA暴露が成長後の情動性に及ぼす影響について特化して検討した結果、乳児期・成体期ともにBPA暴露群において情動性の変化が認められた。

○成体期実験の結果は、BPA暴露の影響が従来の報告よりもはるかに長期にわたることを示唆している。

○成体期の実験において見られた環境反応性の変化は、BPAの胎生期・授乳期暴露による行動影響が中枢だけでなく末梢の変化に起因する可能性を示唆している。

○行動学的解析に加えて電気生理学的手法や免疫組織学的手法を組み合わせ、更に詳細な検討を加える必要がある。

雑誌

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Epigenetic Regulation of Adipogenesis by PHF2 Histone Demethylase

Yosuke Okuno,¹ Fumiaki Ohtake,¹ Katsuhide Igarashi,² Jun Kanno,² Takahiro Matsumoto,¹ Ichiro Takada,¹ Shigeaki Kato,³ and Yuuki Imai¹

PHF2 is a JmjC family histone demethylase that removes the methyl group from H3K9me2 and works as a coactivator for several metabolism-related transcription factors. In this study, we examined the *in vivo* role of PHF2 in mice. We generated *Phf2* floxed mice, systemic *Phf2* null mice by crossing *Phf2* floxed mice with *CMV-Cre* transgenic mice, and tamoxifen-inducible *Phf2* knockout mice by crossing *Phf2* floxed mice with *Cre-ERT2* transgenic mice. Systemic *Phf2* null mice had partial neonatal death and growth retardation and exhibited less adipose tissue and reduced adipocyte numbers compared with control littermates. Tamoxifen-induced conditional knockout of PHF2 resulted in impaired adipogenesis in stromal vascular cells from the adipose tissue of tamoxifen-inducible *Phf2* knockout mice as well as of *Phf2* knocked-down 3T3-L1 cells. PHF2 interacts with CEBPA and demethylates H3K9me2 in the promoters of CEBPA-regulated adipogenic genes. These findings suggest that PHF2 histone demethylase potentiates adipogenesis through interaction with CEBPA *in vivo*. Taken together, PHF2 may be a novel therapeutic target in the treatment of obesity and the metabolic syndrome.

The architecture of eukaryotic chromatin is dynamically modulated by posttranslational modifications of the histones, including acetylation, phosphorylation, ubiquitination, and methylation (1). Methylation states of histones are crucial for chromatin reorganization and regulation of gene transcription. For example, lysine (K) methylation at H3K9, H3K27, and H4K20 is associated with regions of transcriptionally silenced chromatin, whereas methylation at H3K4, H3K36, and H3K79 is associated with transcriptionally active regions. Such modifications are controlled by a balance between enzymes that catalyze the addition and removal of methyl groups. LSD1 and the Jumonji C (JmjC) domain-containing proteins have been shown to possess such histone demethylase activities (2–4).

Plant homeodomain finger 2 (PHF2) is a newly characterized JmjC domain-containing protein identified as an interactant of nuclear receptors. PHF2 forms a complex with the AT-rich interactive domain 5B (ARID5B) and works as a coactivator for farnesoid X receptor (FXR) or hepatocyte nuclear factor 4 α (HNF4A). It is enzymatically

inactive by itself but becomes an active H3K9me2 demethylase through protein kinase A (PKA)-mediated phosphorylation (5).

Although an increasing number of histone demethylases have been identified and their molecular functions progressively unraveled, the physiological functions of these demethylases remain largely unknown. Recently, LSD1 was reported to be required for embryogenesis (6), whereas JHDM2A is required for spermatogenesis (7) and obesity resistance (8) *in vivo*. In zebra fish, PHF8 and KDM7, which belong to the same subfamily of JmjC domain proteins as PHF2, regulate brain development (9,10). It has been suggested that PHF2 plays a role in induction of gluconeogenic genes by PKA signaling in hepatocytes (5) or rRNA expression in nucleoli (11) *in vitro*. However, *in vivo* analyses are required to explore the physiological role of PHF2. In this study, we generated PHF2 knockout mice and found that PHF2 plays a role in both neonatal growth and adipogenesis. These results imply that PHF2 demethylase function would be a novel translational target for human metabolic diseases.

RESEARCH DESIGN AND METHODS

Generation of *Phf2* floxed mice by gene targeting. A bacterial artificial chromosome (BAC) DNA containing mouse *Phf2* (BAC clone RP23-114C14) was obtained from the BAC-PAC Resources Center. *LoxP* was inserted between exons 6 and 7 of *Phf2* using the *Escherichia coli*-based BAC modification system (12). Modification cassettes were generated by PCR amplification of the PL452 vector with the following primers: 5'-TATATAAGGAGCAGCTGGGACCAGT GACATACATGTGTCTAATGTCCTGAGAATTCATTCGATCATATTCATAACCC-3' and 5'-ACTCTGGACACATAGGTGACCCAGTGGCCTCTCCTAATAGTTAATGA GCTCGAACTAGTGGATCCCTCGA-3'. *LoxP*-inserted fragments of mouse *Phf2* were subcloned into the pBSIKS+ vector using the *E. coli*-based BAC recombination system. Modification cassettes and retrieval cassettes were generated by PCR amplification of the pBSIKS+ vector with primers corresponding to each promoter. *LacZ-PGK-Neo* was digested from the pNTR-lacZ-PGK-neo-lox vector and inserted into the pMC1DTpA vector that contained *DT-A*. Oligonucleotides of *loxP* and *frt* were inserted into this vector (cassette vector). *LoxP*-inserted fragments of mouse *Phf2* were inserted into the cassette vector to form the final *Phf2* knockout construct. The *Phf2* knockout construct was linearized by *SacII* and was introduced into M1 mouse embryonic cells (RIKEN) by electroporation and screened by genomic Southern blotting. Chimeric mice were generated by aggregation of embryonic stem cells with eight cell embryos of BDF1 mice. *Phf2*^{+/+} mice were generated by crossing *Phf2*^{+/+} mice with *CMV-Cre* mice (13). *Phf2*^{+/+} mice were generated by crossing *Phf2*^{+/+} mice with Flpe deleter strain ACTB-Flpe mice (Jackson Laboratory). Mice with *Phf2*^{+/+}, *Phf2*^{+/+}, and *Phf2*^{+/+} were maintained by backcrossing to C57BL/6J mice under a specific pathogen-free environment. All animals were maintained according to the protocol approved by the Animal Care and Use Committee of The University of Tokyo.

Generation of *Phf2* conditional knockout mice and genotyping. *Cre-ERT2* transgenic mice were provided by Dr. Daniel Metzger (14). *Cre-ERT2* transgenic mice were crossed with *Phf2*^{+/+} mice to generate *Phf2*^{+/+}*Cre-ERT2* mice. Genotyping was performed by PCR using corresponding primers. Sequences of primers were as follows: P1, 5'-CACCCTCTGTGCTCTCTGT-3'; P2, 5'-CAGTTCTCTTAGCTCCCCCTTT-3'; P3, 5'-GACAGGAAGCCAGGAGGATG-3'; P4, 5'-GACAGCCTGGTCAGGTGAAT-3'; and P5, 5'-TGTAATACACCTGGGGCTCA-3'. *Cre-ERT2* transgenic mice were identified by amplification of the *Cre* allele using primers 5'-TTACGGCGCTAAGGATGACT-3' and 5'-TTGCCCTGTTTCACTATCC-3'.

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Adipose tissue collection. Mice were killed by cervical dislocation. Epididymal, gonadal, lumbar subcutaneous, and mesenteric white adipose tissues (WATs) were carefully separated from testis, uterus, skin, and intestines, respectively. Wet weights of these WATs were measured.

Glucose and insulin tolerance tests. Food was removed 2 h before glucose (2 g/kg) or insulin (1 units/kg) was administered by intraperitoneal injection. Blood samples were collected from the tail vein at various times after the glucose load, as indicated. Plasma glucose was immediately determined on an Accu-Chek Aviva (Roche).

Cell culture. 3T3-L1 mouse fibroblasts were maintained and differentiated into adipocytes as previously described (15). Mouse primary embryonic fibroblasts were isolated on embryonic day 13.5 and cultured in Dulbecco's modified Eagle's medium with 10% FBS.

Plasmids. FLAG-tagged, full-length cDNAs of human CEBPA or PPARG were inserted into pcDNA3 vectors (Invitrogen).

Western blot. Protein levels were determined by blotting with anti-FLAG antibody (Sigma-Aldrich), anti-CEBPA antibody (Santa Cruz Biotechnology), and anti-PHF2 antibody (5). ECL Plus (Amersham) was used for detection of the immunoreactive bands.

mRNA analysis. Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using PrimeScript RT Master Mix (Takara) and subjected to real-time PCR using KAPA SYBR Fast qPCR Kits (Kapa Biosystems) with Thermal Cycler Dice (Takara) according to the manufacturers' instructions. The following primers were used: mouse *Phf2*, 5'-TACTGCCTGATCTGTGTGAAGGA-3' and 5'-CTGGCCGGATGAGATAGAAGA-3'; mouse *Pparg*, 5'-TTA-CTGCCGCTCCACAAAA-3' and 5'-TGAGACATCCCCACAGCAAG-3'; mouse *Cebpa*, 5'-TGCCAACTGAGACTTTCCTACTAAC-3' and 5'-CCCAAAACATCCCTAAACAAAA-3'; mouse *Adipog*, 5'-CAAGGCCGTTCTCTTCCACTAC-3' and 5'-TCCCCATCCCCATACACCT-3'; mouse *Fabp4*, 5'-CAGCGTAAATGGGGATTGG-3' and 5'-GCTCTTACCTTCTCTGTCT-3'; mouse *Plin2*, 5'-GAGCTGGAGATGGAAGCAAAA-3' and 5'-GTGATAAGCCGAGAGCAGAG-3'; mouse *Cd36*, 5'-AAGAACAGCAGCAAAATCAAGG-3' and 5'-AGACAGTGAAGGCTCAAGATGG-3'; mouse *Lpl*, 5'-TGGGACTGAGGATGGCAAG-3' and 5'-GGCAGGTTGAAGGAATGT-3'; and mouse *Arbp*, 5'-GCTCAAGCAGATGCAGCA-3' and 5'-CCGGATGTGAGGCAGCAG-3'.

Retroviral infection. Platinum-E cells were transfected with either pSuper-retro-puro-shLacZ or pSuper-retro-puro-shPHF2 (5'-GCCTGACTCAGTTTCCCAACT-3') using Lipofectamine (Invitrogen). Forty-eight hours after transfection, the medium containing retroviruses was harvested, filtered, and transferred to 3T3-L1 cells. Infected cells were selected with 1 μ g/mL puromycin.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were carried out essentially as previously described (16). For immunoprecipitation, antibody against CEBPA (sc-61; Santa Cruz Biotechnology) or PHF2 (5) was used. The precipitated DNA fragments were amplified by real-time PCR. The following primers were used: mouse *Cebpa*-CEBP, 5'-TTGCGC-CACGATCTCTCTC-3' and 5'-CTTAGAGCCCGCTTCTCTC-3'; mouse *Pparg*-CEBP, 5'-CCACTGGTGTGATTTTACTGCAA-3' and 5'-GTTCTGTGAGGGGCGTGAA-3'; mouse *Fabp4*-CEBP, 5'-CATTGCCAGGGAGAACCAA-3' and 5'-CCATGTGAC-TGTAGGAGTGACCAA-3'; and mouse negative control region, 5'-CAGACATGTCAA-TCAAGAAGACAG-3' and 5'-ACTTTGGAGGAAGAGGCAGAAA-3'.

Microarray analysis. These procedures were conducted according to the PerceLome method (17). Briefly, mRNA expression values were normalized to the cell numbers in each sample by adding external spike mRNAs to them in proportion to the genomic DNA concentration and using the spike RNA quantity data as a dose-response standard curve for each sample. Total RNAs were purified from epididymal adipose tissue using an RNeasy Mini kit (Qiagen). First-strand cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen). After second-strand synthesis, the double-stranded cDNAs were purified using a GeneChip (Affymetrix) Sample Cleanup Module and labeled by in vitro transcription using a BioArray HighYield RNA Transcript Labeling Kit (Enzo Life Sciences). The labeled cRNA was then purified using a GeneChip Sample Cleanup Module. Purified cRNA was hybridized with GeneChip Mouse Genome 430 2.0 Array. Washing and staining were performed in a GeneChip Fluidics Station using the appropriate antibody amplification, washing, and staining protocols. The phycoerythrin-stained arrays were scanned as digital image files, which were analyzed with GeneChip Operating Software. The expression data were converted to copy numbers of mRNA per cell by the PerceLome method and analyzed using PerceLome software.

Morphometric analysis. Paraffin sections of epididymal WAT were processed for hematoxylin-eosin staining and observed by light microscopy. Adipocyte areas were measured using OsteoMeasure.

Evaluation of adipocyte number. The number of adipocytes in epididymal WAT was estimated as previously described (18). Briefly, adipose tissue was minced and fixed at 37°C for 96 h in 1.88% osmium tetroxide. After washing twice with PBS, it was replaced with 8 mol/L urea at room temperature for 48

h. Urea-free particles were washed through a 180- μ m net (Millipore), trapped on a 10- μ m net (Millipore), and suspended using 0.01% Triton X-100 in PBS. Particles were counted with a counting chamber.

Oil red O staining. Cells were stained with oil red O, and the quantification was performed as previously described (19). Briefly, cells were fixed with 10% formalin and stained in a working solution of oil red O. Isopropyl alcohol was added to the stained culture dishes, and the extracted dye was monitored spectrophotometrically at 510 nm.

Statistical analysis. Data were analyzed by two-tailed Student *t* test, Pearson χ^2 test, log-rank test, or two-way repeated-measures ANOVA. For all graphs, data are presented as mean $\pm$ SEM. Statistical significance was accepted at $P < 0.05$.

RESULTS

Generation of *Phf2* floxed mice. Figure 1A shows the strategy to generate the mutant *Phf2* mutant allele. Successful insertion of *LacZ*-*PGK*-*Neo^R* cassettes and *loxP* at sites flanking exon 6 of the *Phf2* allele was confirmed by Southern blotting (Fig. 1B). Cre-mediated recombination excised exon 6 and *PGK*-*Neo^R* from the mutant allele, resulting in the *LacZ* allele. P1, P2, and P3 primers were used to differentiate the wild-type and *LacZ* alleles (Fig. 1C). Flpe-mediated recombination excised *LacZ* and *PGK*-*Neo^R* from the mutant allele, resulting in a floxed allele. P4 and P5 primers were used to differentiate the wild-type and floxed alleles (Fig. 1D). Cre-mediated recombination excised exon 6 from the floxed allele, resulting in a null allele. Exon 6 corresponds to part of the JmjC domain of *Phf2*, and its deletion is expected to result in an alteration of the reading frame of the *Phf2* transcript. As expected, the PHF2 protein was not detected in mouse embryonic fibroblasts of *Phf2^{2/2}* (Fig. 1E).

***Phf2^{2/2}* mice were characterized by partial neonatal death and postnatal growth retardation.** *Phf2^{2/2}* mice were born according to expected Mendelian ratios (Fig. 2A). However, <30% of *Phf2^{2/2}* pups were alive when they reached 2 weeks of age (Fig. 2B). In fact, daily observation revealed that ~70% of *Phf2^{2/2}* mice died within 3 days of birth ($P = 3.0 \times 10^{-5}$ compared with wild type) (Fig. 2C). However, after 3 days of age, the remaining pups retained viability and lived for no less than 6 months (data not shown). *Phf2^{2/2}* mice had progressively reduced growth compared with littermate controls until 10 days after birth, although there was little difference in body weight among newborn mice of different genotypes (Fig. 2D). Two weeks after birth, *Phf2^{2/2}* mice grew in a manner similar to wild-type or heterozygous littermates, but with significant differences in body weight (Fig. 2E). The extent of these weight reductions was not significantly different between males and females (two-way repeated-measures ANOVA). These data suggest that PHF2 is dispensable for the survival of embryos but is necessary for survival and growth in the neonatal period.

Reduced adipose tissue in *Phf2^{2/2}*. Next, we surveyed the weight and appearance of each tissue present in male *Phf2^{2/2}* mice at 5 weeks of age. As mentioned previously, *Phf2^{2/2}* mice had decreased body weights at that time (Fig. 3A). *Phf2^{2/2}* mice exhibited significantly shortened body lengths (Fig. 3A). Various tissues of *Phf2^{2/2}*, including brown adipose tissue (BAT), appeared normal and weighed the same as in control littermates. However, WAT and brain showed a different trend. The brain weights were significantly increased compared with wild-type littermates, the potential significance of which will be discussed later (Fig. 3A). The weight of epididymal WAT was only 50% of that found in control littermates, even when normalized by body weight. Weight reduction was also

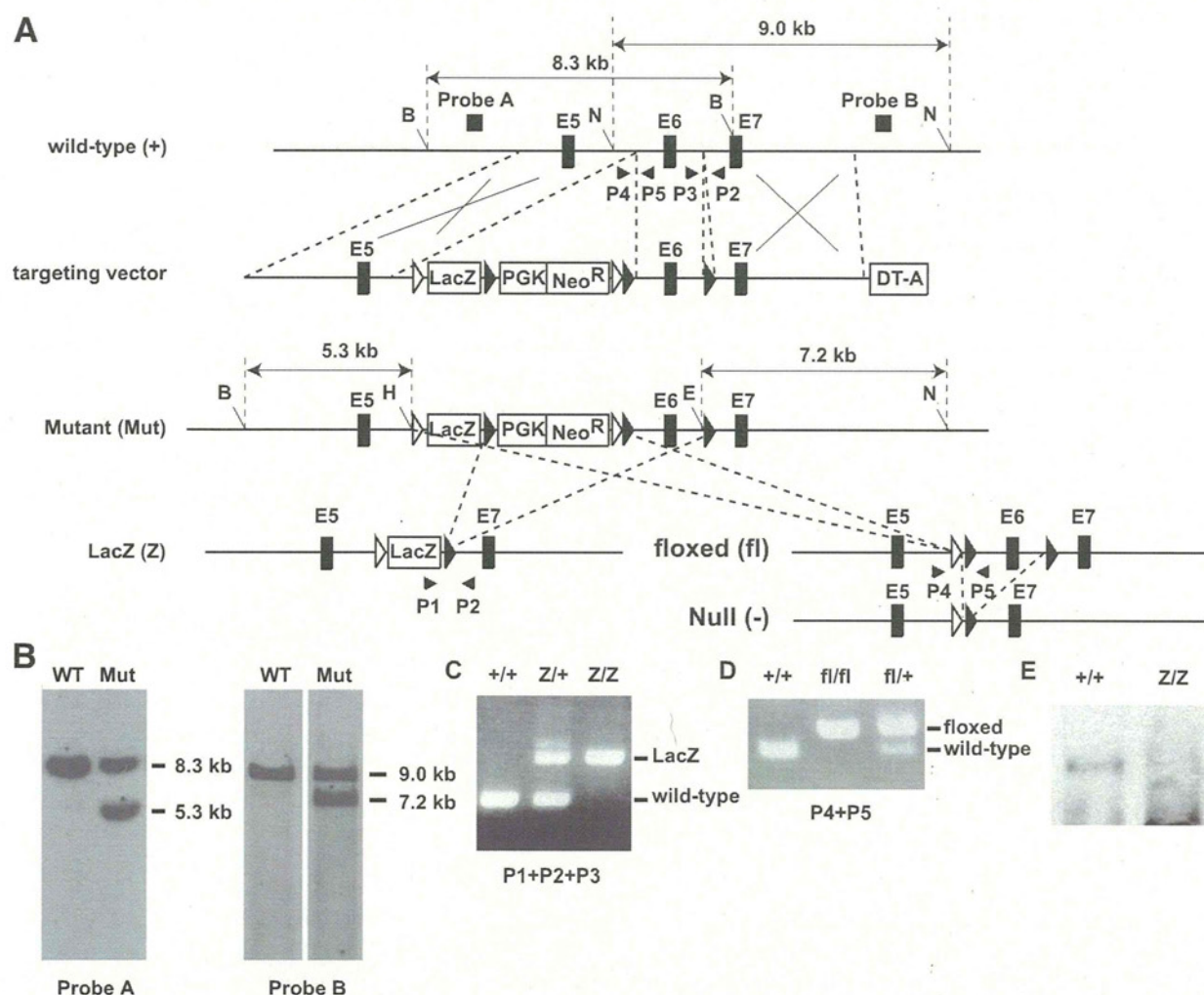


FIG. 1. Strategic scheme for targeted disruption of mouse *Phf2*. **A:** Targeting strategy with positive/negative selection. Strategy of genomic Southern blotting in the screening for homologous recombinant embryonic stem cell clones is also included. E5, E6, and E7 represent exon 5, exon 6, and exon 7 of *Phf2*, respectively. B, H, E, and N represent BglII, HindIII, EcoRI, and NsiI cut sites, respectively. P1, P2, P3, P4, and P5 represent locations of primers used in C and D. Δ , the *LoxP* sites; ∇ , *Frt* sites. **B:** Southern blotting analysis of targeted embryonic stem cell clones. Restriction enzymes used for screening recombination events with probe A were BglII and HindIII. An 8.3-kb fragment in WT and a 5.3-kb fragment after homologous recombination were expected with probe A. Restriction enzymes used for screening recombination events with probe B were EcoRI and NsiI. A 9.0-kb fragment in WT and a 7.2-kb fragment after homologous recombination were expected with probe B. **C:** To detect the presence of the *LacZ* allele (Z) and the WT allele (+), primers P1, P2, and P3 were used. The PCR bands of the WT allele (242 bp) and the *LacZ* allele (495 bp) are indicated. **D:** To detect the presence of the floxed allele (fl) and the WT allele (+), primers P4 and P5 were used. The PCR bands of the WT allele (162 bp) and the floxed allele (245 bp) are indicated. **E:** Western blot analysis of PHF2 protein expression in *Phf2*^{Z/Z} mice. Extracts of mouse embryonic fibroblasts from WT or *Phf2*^{Z/Z} were immunoprecipitated and detected with anti-PHF2 antibody. WT, wild type.

observed in subcutaneous WAT to an extent similar to that in epididymal WAT ($P = 0.06$) but to a lesser extent in mesenteric WAT (Fig. 3B). Additionally, female *Phf2*^{Z/Z} mice exhibited a similar reduced weight of gonadal WAT as observed in male mice (Fig. 3C). However, these weight reductions in adipose tissues seemed to be limited to a young age because there was no significant difference of WAT weights between *Phf2*^{Z/Z} and wild-type littermates when the mice reached 8 weeks of age (Supplementary Fig. 1). To elucidate whether the decreased weight of WAT was caused by a reduction of lipid droplets per cell or adipocyte number, we measured adipocyte size and number in epididymal WAT of *Phf2*^{Z/Z} and wild-type littermates. Evaluation of the mean adipocyte area in the section of WAT revealed that *Phf2*^{Z/Z} mice have smaller adipocytes than do control littermates (Fig. 3D). Furthermore, counting the number of adipocytes in WAT revealed

that *Phf2*^{Z/Z} mice have fewer adipocytes in adipose tissue (Fig. 3E). These data suggest that the decreased weight of WAT in *Phf2*^{Z/Z} mice was caused by a reduction in both the size and the number of adipocytes. Because impaired adipogenesis often results in decreased size (20) and a decreased number of adipocytes, we used microarray analysis to compare the gene expression profiles in WAT between *Phf2*^{Z/Z} mice and control littermates. As a result, the expression of various genes associated with adipogenesis, such as *Pparg*, *Cebpa*, *Fabp4*, *Adipog*, *LPL*, *Plin2*, and *Cd36*, tended to be decreased in *Phf2*^{Z/Z} mice (Supplementary Fig. 2). The expression levels of more than half of these genes were significantly reduced when confirmed by real-time quantitative PCR (qPCR) (Fig. 3F). These data suggest that decreased WAT weight in *Phf2*^{Z/Z} mice resulted, at least in part, from impaired adipogenesis in these mice. Finally, we measured insulin sensitivity in

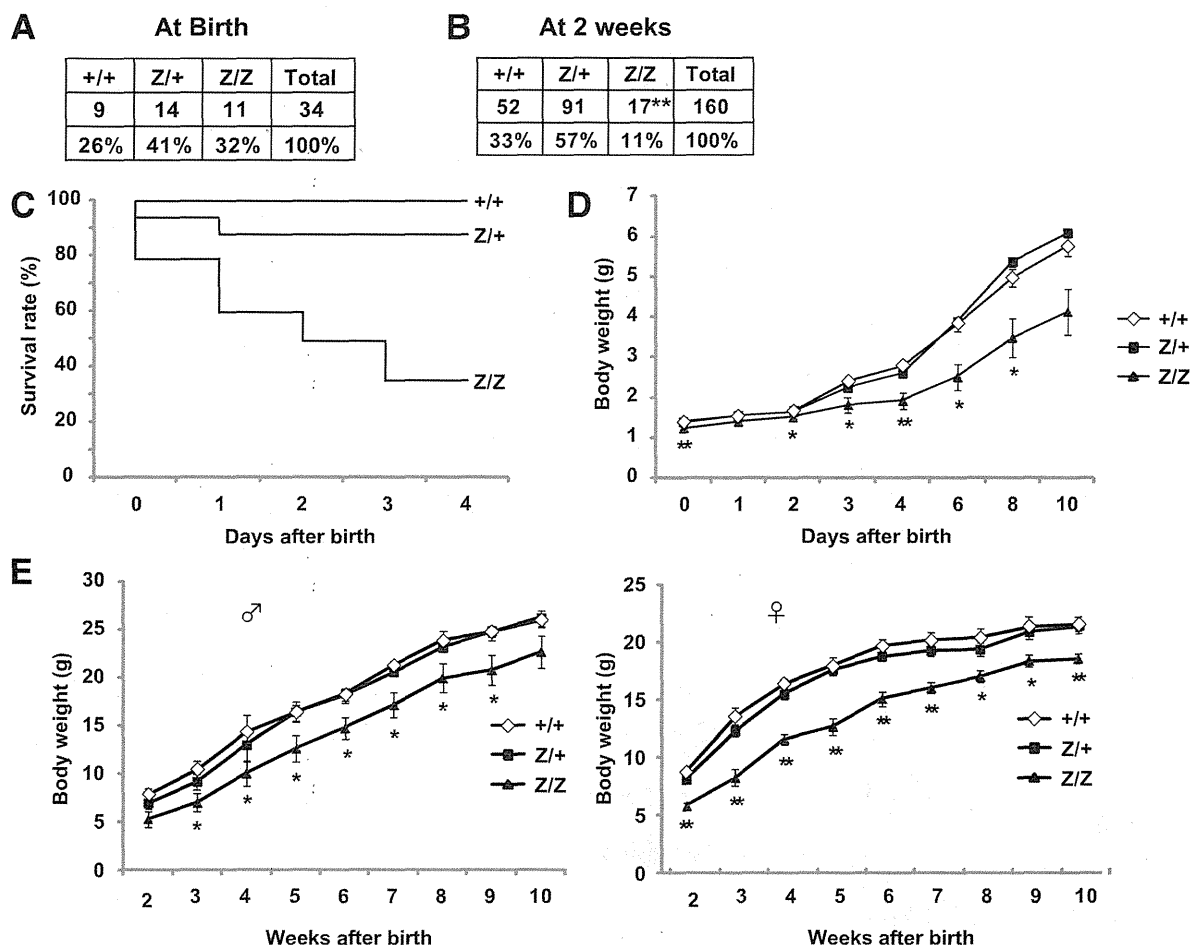


FIG. 2. Physiological features of systemic *Phf2* knockout mice. **A**: Genotypes of progeny of crosses between *Phf2*^{Z/+} at birth. **B**: Genotypes of progeny of crosses between *Phf2*^{Z/+} at 2 weeks of age. **C**: Survival rate of progeny of crosses between *Phf2*^{Z/+}. **D**: Growth curves of wild-type, heterozygous, and homozygous *Phf2* knockout mice until 10 days after birth. **E**: Growth curves of male mice and female mice of indicated genotypes between 2 and 10 weeks of age. ◇, wild-type mice (+/+); ■, heterozygous *Phf2* knockout mice (Z/+); ▲, homozygous *Phf2* knockout mice (Z/Z). **P* < 0.05; ***P* < 0.01 compared with wild type.

Phf2^{Z/Z} mice because adipose tissue is well known to be involved in glucose metabolism. There was no significant change in the glucose or insulin tolerance tests (Supplementary Fig. 3), suggesting that reduced WAT weight did not affect insulin sensitivity in mice at 5 weeks of age.

PHF2 is necessary for normal adipogenesis. To elucidate the role of PHF2 in adipogenesis, we generated *Phf2*^{fl/fl}; *Cre-ERT2* mice by crossing *Phf2* floxed mice with *Cre-ERT2* mice. Stromal vascular cells (SVCs) were then obtained from *Phf2*^{fl/fl}; *Cre-ERT2* mice or control *Cre-ERT2* mice and treated with 4-hydroxytamoxifen (4-OHT) to induce Cre-mediated excision of *Phf2*. As expected, treatment with 4-OHT efficiently reduced mRNA expression of *Phf2* in *Phf2*^{fl/fl}; *Cre-ERT2* but not in control *Cre-ERT2* mice (Fig. 4C). When SVCs were treated with a differentiation cocktail to induce their differentiation into adipocytes, differentiation was impaired in 4-OHT-treated SVCs from *Phf2*^{fl/fl}; *Cre-ERT2* mice as shown by oil red O staining (Fig. 4A and B). This was also confirmed by decreased expression of adipogenic marker genes in 4-OHT-treated SVCs from *Phf2*^{fl/fl}; *Cre-ERT2* mice (Fig. 4C). Next, we generated 3T3-L1 cell lines in which PHF2 was stably knocked down by retrovirus carrying *Phf2*-targeted short hairpin RNA. Infection of retrovirus carrying *shPhf2*

successfully decreased the expression of *Phf2* (Fig. 4F). In accordance with the results from SVCs, short hairpin RNA-mediated knockdown of *Phf2* resulted in impaired adipogenesis in the 3T3-L1 cell line as assessed by oil red O staining (Fig. 4D and E) and expression of adipogenic marker genes (Fig. 4F).

PHF2 is recruited with CEBPA to the promoter regions of adipogenic genes. The results indicated that PHF2 promoted adipogenesis. Moreover, we show that PHF2 works as a coactivator for several transcription factors through H3K9me2 demethylation (5). Therefore, we hypothesized that PHF2 coactivates transcription factors that promote adipogenesis. Among such transcription factors, PPARG and CEBPA are the master regulators for adipogenesis (21). First, we assessed whether PHF2 could physically interact with PPARG or CEBPA. When transfected into HEK293 cells, FLAG-CEBPA (but not FLAG-PPARG) was coimmunoprecipitated with endogenous PHF2 (Fig. 5A). Conversely, FLAG-PHF2 was coimmunoprecipitated with endogenous CEBPA in 3T3-L1 adipocytes (Fig. 5B). In accordance with these results, ChIP analysis revealed that PHF2 was recruited to known CEBPRE (CEBP responsive elements) in the promoter regions of *Cebpa*, *Pparg*, and *Fabp4* in differentiated

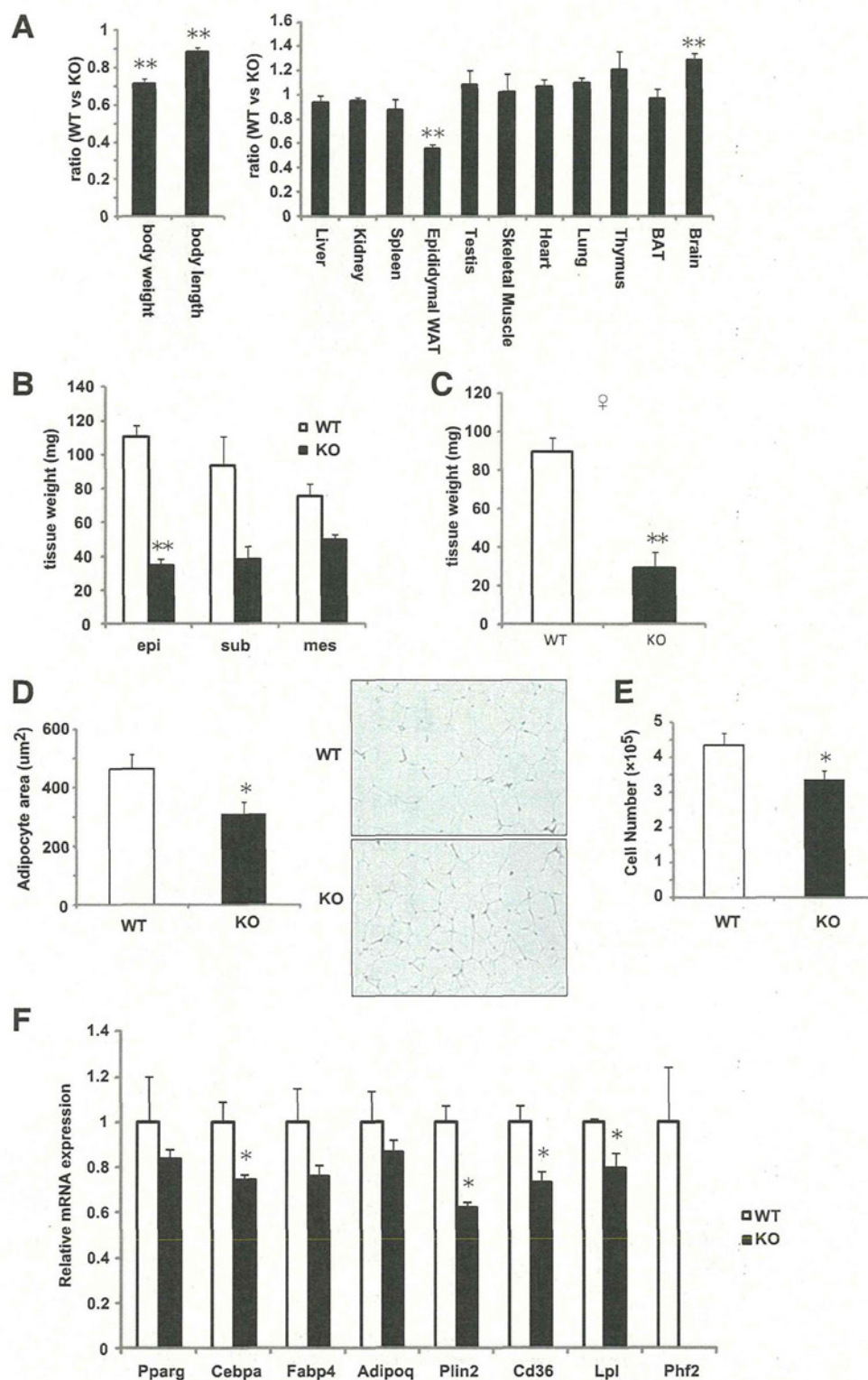


FIG. 3. Assessment of adipose tissue of systemic *Phf2* knockout mice. **A:** Ratio of body weight and nasoanal length or normalized tissue weight of male *Phf2*^{2/2} to WT littermates at 5 weeks of age. Tissue weights were normalized to body weights ($n = 6$). **B:** Weights of epididymal WAT, subcutaneous WAT, and mesenteric WAT of male *Phf2*^{2/2} KO mice and WT littermates at 5 weeks of age ($n = 3$). **C:** Weights of gonadal WAT of female *Phf2*^{2/2} KO mice and WT littermates at 5 weeks of age ($n = 4$). **D:** Mean adipocyte areas of epididymal WAT from *Phf2*^{2/2} KO mice and WT littermates ($n = 5$). High-magnification micrographs of WAT are shown. **E:** Adipocyte number in epididymal fat pads of *Phf2*^{2/2} KO mice and WT littermates ($n = 7$). **F:** Real-time qPCR analysis of adipocyte marker genes and *Phf2* of *Phf2*^{2/2} KO mice and WT littermates ($n = 3$). * $P < 0.05$; ** $P < 0.01$ compared with WT. KO, knockout; WT, wild type. (A high-quality color representation of this figure is available in the online issue.)