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ヒトへの外挿を目指したナノマテリアルの健康影響評価手法の開発

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（H24－化学－一般－004）総括研究報告書

「ヒトへの外挿を目指したナノマテリアルの健康影響評価手法の開発」に関する研究

研究代表者:武田　健（東京理科大学 総合研究機構 教授）

研究要旨： 本プロジェクトではげっ歯類ならびに霊長類モデルを用いて、ナノマテリアルの妊娠期曝露による次世代の免疫系、中枢神経系、雄性生殖系に生じる影響の詳細を検証した。これにより、ナノマテリアル（低用量曝露を含む）の次世代影響に焦点を当てたリスク評価法を確立することを目指した。具体的には、①低用量のナノマテリアルによる次世代雄性生殖系への影響評価、②ナノマテリアルの妊娠期曝露による次世代免疫系・中枢神経系への影響評価指標探索、③ナノマテリアルによる霊長類リンパ節・中枢神経系への影響評価指標探索を目的として研究を進めた。最終年度に二年度目までの結果を総括しながら、In vivo だけでなく ex vivo のナノマテリアル健康影響評価系の構築を目指した。

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A. 研究目的

本研究課題は、ナノマテリアルの健康影響について特に、1) 低用量曝露による次世代雄性生殖系への影響、2) 次世代の免疫系・中枢神経系への影響、3) 霊長類免疫系・中枢神経系に対する影響を明らかにしようとしたものである。1) では妊娠期におけるナノマテリアルの低用量曝露が次世代に及ぼす影響について、次世代の雄性生殖系機能をマーカーとしたげっ歯類の影響評価系の確立を目指した。2) 3) を通して、ナノマテリアルが免疫系に及ぼす影響について、ヒトに外挿できる霊長類及びげっ歯類の評価系を確立することを目指した。

B. 研究方法

<u>げっ歯類（マウス）を用いた研究</u>

妊娠マウス（ICR 系、C57BL/6 系）にナノマテリアルを投与した。材料は、生産量が多く汎用されているもののうち、カーボンブラックおよび酸化チタンを用いた。とくに酸化チタンは、アルミナコーティングにより表面性状の異なるものの影響を比較検討した。酸化チタンナノ粒子は懸濁液を皮下、炭素（カーボンブラック）ナノ粒子は懸濁液を気道（点鼻）に投与した。なお、懸濁媒中でのナノ粒子の存在状態は透過及び走査型電子顕微鏡、ならびに動的光散乱法（DLS）により解析した。その結果、とくに低濃度

（低用量投与）のナノ粒子懸濁液については、マテリアルが二次粒子径も 100nm 未満というスケールに収まっていることを確認した。投与したナノ粒子の動態については、とくに胎仔への移行を透過型電子顕微鏡ならびに走査型電子顕微鏡－エネルギー分散型X線スペクトロ測定装置（FE-SEM/EDS）により解析した。

次世代雄性生殖器への影響は、母体に投与したナノ粒子の移行・蓄積ならびに精子・精巣の超微小形態の観察により評価した。次世代免疫系への影響は、フローサイトメトリーを用いたリンパ球組成の解析と遺伝子発現解析（機能的トランスクリプトミクスならびに定量的 RT-PCR）により評価した。併せて、産仔の免疫系組織及び血液中 miRNA の網羅的解析を行った。さらに、二酸化チタンナノ粒子の次世代影響標的として有力な脳において、影響発現メカニズムをエピジェネティクスの観点から明らかにするために、脳組織における DNA メチル化プロファイルを網羅的に解析した。

（倫理面への配慮）

げっ歯類動物実験は、東京理科大学倫理委員会での承認を得、文部科学省「研究機関等の動物実験等の実施に関する基本指針」、東京理科大学動物実験指針を遵守して行った。ナノ粒子の安全性が不明であることから、P2 プラスレベルの実験に準じた作業手順を実施した。

霊長類（サル）を用いた研究

アカゲザル新生仔及び成体の背部皮内に、ナノ材料としてディーゼル排気ナノ粒子（DEP）、非金属ナノ粒子（カーボンブラック：CB）、酸化金属ナノ粒子（二酸化チタン：TiO_2）、蛍光ナノポリスチレン（PS）のいずれかをそれぞれ投与し、1ヶ月～3 年後に投与部位、リンパ節、主要組織の試料を採取した。試料から RNA を抽出した後、遺伝子の発現変動をマイクロアレイ及び定量的 RT-PCR により解析し、その機能的特徴を明らかにした。

また、胎仔期暴露の影響を調べるため、妊娠アカゲザルの背部皮内に、DEP、CB、 TiO2 のいずれかを投与し、それらの新生仔の脳を含む主要組織を採取した。遺伝子及びタンパク質の発現変動をマイクロアレイ、RT-PCR、ウェスタンブロット、組織免疫染色により解析した。

（倫理面への配慮）

サルモデルでの実験は NPO 法人プライメイト・アゴラ動物実験倫理委員会での承認を得て実施した。その際、動物福祉に関しては同法人バイオメディカル研究所動物実験指針に準拠した。また、投与試験に関しては、環境汚染対応が可能な飼育・実験室で実施した。

C. 研究成果

1）次世代雄性生殖系への影響

前年度に続いて、銀ナノ粒子の妊娠期飲水投与が次世代雄性生殖系に及ぼす影響を検証した。まず、懸濁液中の銀ナノ粒子を低濃度の塩により凝集させ、二次粒子径を大きくした（>200 nm）場合でも、次世代雄性生殖系に及ぶ影響は消失しなかった。また、投与時期による影響発現を差異を検証する目的で、一部のマウスに対し出生後にも銀ナノ粒子を投与し、銀ナノ粒子の毒性発現に重要な曝露時期の検証を進めている。

銀ナノ粒子を思春期のみ投与した群に比べ胎児期のみ投与した群 （Ag-Pre

群）では精巣上体重量が、胎児期および思春期に投与した群（AgH 群）では精巣重量並びに精巣上体重量が有意に低下した。また、精細管の各構成細胞の細胞数を測定した結果、AgH 群のセルトリ細胞と精母細胞、Ag-Post 群のセルトリ細胞の細胞数が対照群と比較して有意に減少していた。

2）次世代免疫系・中枢神経系への影響

前年度までに引き続き、カーボンブラックナノ粒子の妊娠前期投与が次世代免疫系に及ぼす影響を検証した。その結果、次世代免疫系（脾臓リンパ球 phenotype）に対する影響発現は、一般急性毒性（気管支肺胞洗浄 BALF 中細胞数増加）の生じるレベルであっても低用量で消失することが明らかになった。

一方で、同用量カーボンブラックナノ粒子の妊娠後期投与は、次世代免疫系（脾臓）に対し T 細胞等の細胞数を著しく増加させることが明らかになった（El-Sayed & Umezawa et al. *Toxicology* 2015）。

次世代中枢神経系に生じる影響について、ナノマテリアルのハザード分類・リスク評価に資する鋭敏かつ定量的なマーカーが得られた。ナノ粒子（カーボンブラック、二酸化チタン）を妊娠期に経気道投与すると、次世代個体の脳血管周囲の細胞（血管周囲マクロファージ PVM ならびにアストロサイト）が鋭敏に反応することが明らかになった（Onoda & Umezawa et al. *PLoS One* 2014）。その作用の程度は、二次粒子径を同程度にしたナノマテリアルで比較した場合、カーボンブラックの方が二酸化チタンナノ粒子に比して大きかった（**国際会議 Nanotoxicology 2014, Best Poster Award**）。また、この影響は大脳皮質前頭野の GFAP タンパク質発現解析（ウェスタン・ブロット法）により定量的に分析することが可能になり、ナノマテリアルの妊娠期経気道投与が次世代中枢神経系に及ぼす影響についての用量依存性も確認することができた。ナノマテリアルの曝露によるヒト健康影響に関して、とくに次世代影響・発達毒性の考慮は、免疫系以上に中枢神経系に及ぶ影響について考慮する必要があるようである。

また、ナノマテリアルの妊娠期経気道投与により次世代中枢神経系に及ぶ影響を、遺伝子発現を制御する DNA メチル化プロファイルから検証した。その結果、*Pcdh9* などの複数の遺伝子を標的として、プロモーター領域の DNA 脱メチル化に伴う遺伝子発現亢進が認められた。

3）霊長類を用いた検討

サル類とげっ歯類との比較発現解析から、リンパ節／免疫系ならびに中枢神経系への影響の分子機序を検討した。さらに、霊長類（ヒトを含む）での ex vivo あるいは semi-in vitro でのナノマテリアル影響評価系についても予備検討した。

その結果、ナノマテリアル投与が免疫系において 2 型マクロファージ（M2 マクロファージ）のマーカー遺伝子であるキチナーゼ（Chitinase 1, CHIT1）或いは CCL18（霊長類特異的ケモカイン）の遺伝子発現亢進が確認された。それらの遺伝子発現亢進は少なくとも 3 年間継続していた。また、サル・ラット双方において、ナノマテリアルが Dendritic cell /Macrophage の T-cell 活性化制御因子である DC-Hil/GPNMB を顕著に発現亢進することを新たに見出した。ナノマテリアルが T-Cell レベルの免疫応答にも影響することが明らかになった。

前述のげっ歯類のデータを踏まえ、ナノマテリアル投与がリンパ節だけでなく中枢神経系（大脳皮質前頭野、小脳、海馬）に及ぼす影響を遺伝子発現変動プロファイリングにより検証した。その結果、胎仔期にナノマテリアルに暴露された新生仔の中枢神経系において複数のナノマテリアル・複数の脳領域に共通する変動パスウェイとして、ヘモグロビン代謝経路が見出された。

さらに、上記霊長類免疫系で得られたデータに基づき、ヒト細胞株を用いたナノマテリアルの影響評価系の確立を目指している。免疫系に及ぶ影響評価については、単球ならびにリンパ球の単培養では in vivo で認められるCHIT1或いはCCL18の発現変動が生じなかった。これは M2 マクロファージ分化誘導について調べるためには、貪食能をある程度高めたマクロファージを用いて実験する必要性を示唆している。生体では、貪食能を持つ Dendritic cell 等マクロファージ系細胞がナノマテリアルを取り込み、多種類の細胞が相互作用しながら応答していると考えられる。ナノマテリアルの免疫系への影響を検討する際には、in vivo の状態を部分的にでも模倣するモデル系を作製する必要性がある。そのため、サイトカイン或いはフォルボールエステルのような化学物質で刺激した単球系細胞を用いたナノマテリアル影響評価系の検討しているが、課題が残った。

D. 考察

「次世代雄性生殖系への影響解析」では、妊娠期マウスに投与した極めて低用量の酸化チタンを次世代の精巣組織内に検出できることを示した。

その上で実験（1）の結果は、投与時期（出生前＝妊娠期／出生後）により銀ナノ粒子の影響する細胞種が異なる可能性を示唆している。また、銀ナノ粒子の毒性発現（とくに発達毒性）は二次粒子径に依存しなかった。ナノマテリアルの体内動態は、凝集状態が変わり二次粒子径が異なれば体内動態が異なるが、少なくとも溶解性のあるナノマテリアルについては、毒性発現プロファイルに対する体内動態の寄与がそれほど大きくない可能性がある。ナノマテリアルの毒性を考える上で、細胞への取り込みを介した直接的な細胞毒性以外によらない間接的なメカニズムの考慮が、ナノマテリアルの曝露によるヒト健康影響評価において重要であると考えられる。

実験（2）の結果、ナノマテリアルの妊娠前期により次世代免疫系に生じる影響は、一般急性毒性の認められる用量に限られることから、ヒト健康影響防止を考える上で考慮に入れなくていいものと考えられる。ただし、ナノマテリアル曝露により次世代に生じ得る健康影響は、妊娠中の曝露時期により大きく異なる可能性があることには注意が必要である。

ナノマテリアル経気道投与により次世代中枢神経系に生じる影響については、前述のとおり感受性高くかつ定量性のある影響評価指標の存在を明らかにすることができた。また、脳に生じる遺伝子発現変動が、げっ歯類と霊長類との間で保存性の高いプロモーター領域の DNA メチル化変動により制御されることが明らかになり、げっ歯類を用いた試験系によりヒトへの外挿性も高く評価できる健康影響評価系と言えると考えられる。

本研究の大きな特色は、ヒトに外挿可能なリスク評価系を確立するために、霊長類モデルを活用した点である。実験（3）では、ナノマテリアル投与により免疫系で M2 マクロファージマーカー遺伝子

発現が亢進することが示された。更に、投与から 3 年を経てもなおナノマテリアルがリンパ節に蓄積したままであり、これらの遺伝子発現が継続的に亢進しているが明らかとなった。M2 マクロファージはアレルギー応答に関与していることが知られている。このため、ナノマテリアルに暴露されたヒトが、長期的にアレルギー疾患発症或いは増悪のリスクを負う危険性が示唆された。リンパ節において変動した遺伝子は、霊長類特異的であるものが多かったが、中枢神経系においてはげっ歯類と共通する点が見出される傾向がある。霊長類の妊娠期ナノマテリアル暴露による産仔中枢神経系におけるヘモグロビン遺伝子発現亢進は、胎仔脳が酸化ストレス或いは低酸素状態に応答していることを示すものでる。ヘモグロビン亢進は細胞障害を起こすことが知られているため、ナノマテリアルによる胎仔中枢神経系発達障害、高次脳機能障害の危険性を示唆している。霊長類胎盤透過性はげっ歯類のそれよりも高く、母子間の物質交換は密であり、妊娠期間も長い。よって霊長類におけるナノマテリアル暴露影響はより重篤であることも推察されるが、げっ歯類モデルにおいても胎仔期ナノマテリアル暴露による中枢神経系のアポトーシスが報告されている。これらのことから、霊長類モデル及びげっ歯類モデルのそれぞれの特長を踏まえ、双方よりヒトへの外挿性を検討することが重要である。中枢神経系への着目は、本プロジェクトの後半からであったためトランスクリプトームデータを検証し切れていないが、このデータの検証を完了させることにより、マウスとサルにおいて妊娠期ナノマテリアル投与により変動した遺伝子群の相同性を検証することにより、げっ歯類の脳のデータのヒト外挿性についての結論が得られると期待している。

E. 結論

本研究課題では、ナノマテリアルの健康影響について、1) 次世代雄性生殖系への影響解析、2) 次世代免疫系・中枢神経系への影響解析を通して次世代影響の短期間（新生児マウス）での評価、ならびに、3) 霊長類免疫系・中枢神経系に及ぼす影響解析を通して、ヒトへの外挿が可能な低用量ナノマテリアルによる影響の鋭敏かつ定量的な評価手法の開発を目指した。

本研究においてヒト細胞株を用いた実験では、in vivo（サル）で認められるナノマテリアル曝露の影響が検出できなかった。免疫系影響評価のためには貪食能を高めたマクロファージ系細胞を用いた評価系の有用性が示唆された。

一方で、発達精巣毒性をはじめとするナノマテリアルの次世代影響は、細胞への取り込みを介した直接的な細胞毒性以外によらない間接的なメカニズムが大きいようである。次には例えば、ナノマテリアルにより生じるタンパク質の高次構造変化と、それに起因する間接的生体影響評価系の構築を指向した研究などが、培養細胞系もしくは cell-free system でのナノマテリアル健康影響評価系の構築に力を発揮するであろうと期待される。

本プロジェクトの成果を考慮に入れた、ナノマテリアルのヒト健康影響評価（胎児や新生児という高感受性集団への影響評価を含む）法は、国民の健康・安全を守ることとナノテクノロジー産業の健全な発展との両立に貢献できると期待している。

なお、我々は 2014 年 9 月 6 日に、**日本学術会議トキシコロジー分科会シンポ**

ジウム「PM2.5 とナノ粒子—微小粒子の健康影響とその対策を考える—」を開催し、本研究課題の経過についても報告し、研究成果の普及（社会還元）に向けた議論を実現した。

F. 研究発表

1．論文発表

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H. 知的財産権の出願・登録状況

なし

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Original Article

Prenatal diesel exhaust exposure disrupts the DNA methylation profile in the brain of mouse offspring

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ABSTRACT — Prenatal diesel exhaust (DE) exposure is associated with detrimental health effects in offspring. Although previous reports suggest that DE exposure affects the brain of offspring in the developmental period, the molecular events associated with the health effects have largely remained unclear. We hypothesized that the DNA methylation state would be disrupted by prenatal DE exposure. In the present study, the authors examined the genome-wide DNA methylation state of the gene promoter and bioinformatically analyzed the obtained data to identify the molecular events related to disrupted DNA methylation. Pregnant C57BL/6J mice were exposed to DE (DEP; 0.1 mg/m^3) in an inhalation chamber on gestational days 0-16. Brains were collected from 1-day-old and 21-day-old offspring. The genome-wide DNA methylation state of the brain genome was analyzed by methylation-specific DNA immunoprecipitation and subsequent promoter tiling array analysis. The genes in which the DNA methylation level was affected by prenatal DE exposure were bioinformatically categorized using Gene Ontology (GO). Differentially methylated DNA regions were detected in all chromosomes in brains collected from both 1-day-old and 21-day-old offspring. Altered DNA methylation was observed independently of the presence of CpG island. Bioinformatic interpretation using GO terms showed that differentially methylated genes with CpG islands in their promoter were commonly enriched in neuronal differentiation and neurogenesis. The results suggest that prenatal DE exposure causes genome-wide disruption of DNA methylation in the brain. Disrupted DNA methylation would disturb neuronal development in the developmental period and may be associated with health and disease in later life.

Key words: Brain, DNA methylation, Diesel exhaust, Prenatal exposure

INTRODUCTION

Epidemiological and experimental studies have shown that exposure to fine ambient particulate matter (PM) is related to respiratory and cardiovascular disorders (Pope *et al.*, 2004; Liu *et al.*, 2008; Ostro *et al.*, 2006). Diesel exhaust (DE) is one of the main types of air pollution and is a major source of fine ambient PM in urban environments (Donaldson *et al.*, 2005). For this reason, DE exposure models have been used to investigate the health effects of ambient PM. Several studies have indicated that DE exposure may affect the central nervous system. For instance, railroad workers exposed to DE have shown neurobehavioral impairment (Kilburn, 2000), and human volunteers exposed to DE have shown altered electrical signals in the frontal cortex (Crüts *et al.*, 2008). Previously, we reported that prenatal DE exposure affects the brain of offspring with regard to neurotransmitter levels and spontaneous locomotor activity (Suzuki *et al.*, 2010). Other studies have shown that prenatal DE exposure induces neuroinflammation and affects behavior in mouse offspring (Bolton *et al.*, 2012; Thirtamara Rajamani *et al.*, 2013). Peters *et al.* (2013) indicated that prenatal DE exposure increases the risk of childhood brain tumors. Although these reports suggest that DE exposure affects the brain of offspring in the developmental period, the molecular event involved in these health effects has largely remained unclear.

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DNA methylation is a critical mechanism of epigenetic gene regulation (Deaton and Bird, 2011). In mammals, methylation almost exclusively occurs on the cytosine residue of CpG dinucleotide. CpG islands are GC-rich DNA regions that possess relatively high densities of CpG dinucleotide. They are found in many genes, positioned mainly at the 5' ends. Their methylation state is closely associated with gene transcription activity. DNA methylation results in transcriptional silencing, either by interfering with a transcription factor binding or by inducing heterochromatin structure. DNA methyltransferases, DNMT1, DNMT3a and DNMT3b, are responsible for the methylation of cytosine residue in CpG sites (Bergman and Cedar, 2013). DNMT3a and DNMT3b play crucial roles in *de novo* cytosine methylation, while DNMT1 works by maintaining the DNA methylation pattern in the newly synthesized DNA during cell division. Knockout studies of DNA methyltransferases have shown that DNA methylation is essential for complete embryonic development (Li *et al.*, 1992; Okano *et al.*, 1999).

In the developmental period, the DNA methylation pattern derived from germ cells disappears when the fertilized egg develops into a blastocyst. The *de novo* methylation pattern is then reestablished at around the stage of implantation (Kafri *et al.*, 1992). The global DNA methylation level also changes in the early postnatal stages (Tawa *et al.*, 1990). This dynamic regulation of the DNA methylation state during the developmental period would provide a mechanism for the removal of errors in gene methylation patterns derived from germ lines; thus allowing normal development. These DNA methylation processes during development are associated with long-lasting phenotypic changes, including genomic imprinting, cell differentiation, and X-chromosome inactivation (Roth *et al.*, 2009). Previous studies indicated that dysregulation of DNA methylation contributes to neuronal disorders such as Rett syndrome, fragile X mental retardation and immunodeficiency, centromeric region instability, and facial anomalies syndrome (ICF) (Amir *et al.*, 1999; Sutcliffe *et al.*, 1992; Tucker, 2001). These phenomena led the authors to suspect that prenatal DE exposure disrupts the DNA methylation pattern and subsequently induces neuronal defects in the brain of offspring.

In the present study, we analyzed the effect of prenatal DE exposure on the DNA methylation state in the brain of offspring mice. The methylation state of the promoter DNA region throughout the entire genome was analyzed with a combination of methylated DNA immunoprecipitation (MeDIP) and promoter tilling array analysis. The differentially methylated genes were then categorized bioinformatically using Gene Ontology (GO) to identify the molecular events associated with altered DNA methylation induced by prenatal DE exposure. Furthermore, Medical Subject Headings (MeSH) analysis was used to identify the brain regions preferentially affected by DE exposure.

MATERIALS AND METHODS

Animals and exposure

C57BL/6J mice were purchased from SLC (Shizuoka, Japan) and housed under controlled conditions (temperature: 22 ± 1°C, humidity: 50 ± 5%) with a 12 hr light/12 hr dark cycle and *ad libitum* access to food and water. Pregnant mice were exposed to DE in an inhalation chamber for 8 hr per day (10:00-18:00) from gestational day 0-16 (DE exposed-group). Pregnant mice of the control group were housed in a clean air chamber. After delivery, offspring were housed in clean air. All experiments were handled in accordance with the institutional and national guidelines for the care and use of laboratory animals.

Diesel exhaust

A four-cylinder 2,179 cc diesel engine (Isuzu Motors, Tokyo, Japan) was operated at a speed of 1,500 rpm and 80% load with diesel fuel. The exhaust was introduced into a stainless steel dilution tunnel (216.3 mm diameter × 5250 mm) where the exhaust was mixed with clean air. The mass and number concentrations of DEP were measured by a Piezobalance Dust Monitor (model 3521, Kanomax Japan, Osaka, Japan) and a condensation particle counter (model 3007, TSI Inc., Shoreview, MN, USA), respectively. Concentrations of gas components, (nitric oxide [NO_X], SO_2, and CO) in the chambers were measured by a NO-NO_2-NO_X analyzer (model 42i; Thermo Fisher Scientific, Franklin, MA, USA), an Enhanced Trace Level SO_2 Analyzer (model 43i-TLE; Thermo Fisher Scientific), and a CO Analyzer (model 48i; Thermo Fisher Scientific), respectively.

DNA extraction

Brain tissues were removed from 1d and 21d offspring of each dam (1d: 6 dams/group, 21d: 5 dams/group). The whole brain tissues obtained were homogenized in extraction buffer (10 mM Tris-HCl [pH8.0], 0.1 M EDTA, 0.5% SDS, 0.3 mg/mL Proteinase K) and incubated at 55°C overnight. After RNase A treatment, genomic DNA was extracted by phenol/chloroform extraction, followed by ethanol precipitation. Extracted DNA of 1 male and 1 female from each dam was pooled for use in DNA methylation analysis. PCR amplification of genomic sequence of sex determining region (*Sry*) gene, which

is located on the Y chromosome, was performed to discriminate the sex of offspring.

Methylated DNA immunoprecipitation (MeDIP)

MeDIP was performed as previously described (Weber *et al.*, 2005), with slight modifications. Before MeDIP assay, genomic DNA was sonicated to produce a random fragment, which mainly ranged from 500-1,000 bp. The fragmented DNA was denatured for 10 min at 95°C and subsequently incubated with anti-5-methyl cytosine antibody (Diagenode Inc., NJ, USA) in immunoprecipitation (IP) buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) overnight at 4°C. Then, the mixture was incubated with Dynabeads M-280 Sheep anti-mouse IgG for 2 hr at 4°C. After washing with IP buffer, the beads were incubated with elution buffer (25 mM Tris-HCl [pH8.0], 10 mM EDTA, 0.5% SDS, 0.25 mM DTT) for 15 min at 65°C. Eluted DNA was recovered by phenol-chloroform extraction followed by ethanol precipitation.

In vitro transcription

T7 based amplification of methylated DNA obtained by MeDIP assay was performed as previously described (Liu *et al.*, 2003), with slight modifications. DNA samples were treated with 5 units of Antarctic phosphatase (New England BioLabs, Ipswich, MA, USA). Poly T tails were generated using terminal transferase (New England BioLabs) with 100 µM dTTP and 10 µM ddCTP. The reaction was cleaned up with a MinElute Reaction Cleanup kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. The tailing reaction product was mixed with T7-polyA primer (5'- GCATTAGCGGCCGCGAA ATTAATACGACTCACTATAGGGAG$(A)_{18}$C-3') in the reaction buffer (10 mM Tris-HCl [pH 7.9], 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.2 mM dNTP mix) and incubated at 95°C for 2 min to denature. After incubation, the reaction was held at 37°C for 2 min to anneal, ramped down to 25°C and held while Klenow enzyme (New England BioLabs) was added. The sample was then incubated at 37°C for 90 min for extension. The reaction was terminated by adding 0.5 M EDTA (pH 8.0), then cleaned up with MinElute Reaction Cleanup kit (Qiagen). After the samples were condensed with a centrifugal vacuum evaporator, *in vitro* transcription was performed using a Megascript T7 kit (Ambion, Foster, CA, USA) according to manufacturer's instructions, with the exception that the incubation was increased to 16 hr. The resultant samples were purified with NucleoSpin RNA Clean-up kit (Machery-Nagel, Düren, Germany).

Biotin-labeled double-stranded cDNA synthesis and promoter tiling array analysis

To obtain double-stranded cDNA, amplified RNA was reverse transcribed, nick-translated and subsequently biotin-labeled with GeneChip WT Double-Stranded DNA Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA) according to manufacturer's instructions. Labeled double-stranded cDNA was used for promoter array analysis.

GeneChip mouse promoter 1.0R array (Affymetrix) was hybridized with biotin-labeled double-stranded cDNA using a GeneChip Hybridization, Wash and Stain kit (Affymetrix). The array was then scanned by Affymetrix GeneChip Command Console software. Labeling, hybridization and scanning were performed by Bio Matrix Research, Inc. (Chiba, Japan) using the standard protocol. Tiling Analysis Software (TAS, Affymetrix), was used to calculate signal intensities, normalize the signals, and generate p-values. The threshold setting for the analysis was set at $p < 0.01$. The detected DNA regions were annotated to genes using the probe information provided by Affymetrix. To analyze whether these genes have CpG islands, the genomic location of CpG islands in the entire mouse genome was obtained from the UCSC genome browser (Center for Biomolecular Science and Engineering, Santa Cruz, CA, USA).

Bioinformatic analysis of methylated genes with GO and MeSH

Differentially methylated genes were categorized with GO and MeSH terms. All promoter DNA regions analyzed by promoter tiling array were annotated with GO using an annotation file (ftp://ftp.ncbi.nih.gov/gene/DATA/gene2go.gz) provided by the NCBI. All of the genes were annotated to MeSH terms using the Gene2MeSH programmatic interface provided by National Center for Integrative Biomedical Informatics (Ann Arbor, MI, USA). The annotations were updated in October 2013. The genes in which the DNA methylation level was affected by prenatal DE exposure were categorized using GO terms. Gene promoters with CpG islands were analyzed because the presence of a CpG island is crucial for transcriptional regulation. The enrichment factor for each category was defined as $(nf/n)/(Nf/N)$, where nf is the number of differentially methylated genes within the category; Nf is the total number of genes within that same category; n is the number of differentially methylated genes within the entire tiling array; and N is the total number of genes on the tiling array. Statistical analysis was performed using Fisher's exact test based on a hypergeometric distribution. Then the GO categories with enrichment

factors ≥ 2, *nf* > 5 and $p < 0.05$ were extracted.

RESULTS

Diesel exhaust characteristics

The average number and mass concentration of the diesel exhaust particles (DEP) were approximately 7.1×10^4 number/cm^3 and 0.1 mg/m^3, respectively. The average concentration of exhaust gases was maintained at 1.84 ppm for carbon monoxide (CO), 0.456 ppm for nitrogen monoxide (NO), 0.247 ppm for nitrogen dioxide (NO_2), and 3.52 ppb for sulfur dioxide (SO_2) (Table 1).

Genome-wide analysis of the promoter DNA methylation state

Probes on the promoter tiling array were annotated to 14,246 genes. The methylated promoter DNA regions specific to the control group and the DE-exposed group were defined as follows. The regions specific to the control group were those in which DNA methylation level in the DE-exposed group was decreased in comparison to the control group. The regions specific to the DE-exposed group were those in which DNA methylation level in the DE-exposed group was increased in comparison to the control group. These methylated promoter DNA regions were detected in all chromosomes (Figs. 1, 2; Supplementary Tables 1-4). We detected 3197, 3010, 3340 and 2820 genes with differentially methylated DNA regions in their promoter in 1-day-old (1d) male, 1d female, 21d male and 21d female offspring, respectively (Figs. 1, 2). Among these genes, 1689, 1905, 1760 and 1522 genes, respectively, have CpG islands (Fig. 1). These results showed that the differential methylation of the gene promoters occurred independently of the presence of CpG islands. Furthermore, altered DNA methylation was detected in both the 1d and 21d offspring. These results indicated that prenatal DE exposure disrupted the genome-wide DNA methylation state in the brain of offspring mice throughout the 1-21-day postpartum period.

Categorization of differentially methylated genes with GO and MeSH

Of the genes mapped to the promoters that were differentially methylated in the DE-exposed and control groups, 57, 74, 98 and 52 GO categories were enriched in 1d male, 1d female, 21d male and 21d female offspring, respectively (Supplementary Tables 5-8). To obtain information about the biological function affected by altered DNA methylation in each time point, GO terms that were common between male and female offspring were extracted (Tables 2, 3). The GO terms related to neuronal differentiation ("positive regulation of neuron differentiation") and neurogenesis ("positive regulation of neurogenesis" and "neurogenesis") were found in both 1d and 21d offspring, respectively.

We also attempted to find the brain regions that were susceptible to prenatal DE exposure from the MeSH results. Although some MeSH terms indicate the brain regions that were enriched in each experimental group, we were not able to find MeSH terms common to each experimental point (Supplementary Tables 9-12).

DISCUSSION

Human epidemiologic and animal studies indicate that nutrition and environmental stimuli during prenatal and postnatal mammalian development influence developmental pathways and thereby induce permanent changes in metabolism and chronic disease susceptibility (McMillen and Robinson, 2005). Epigenetic mechanisms are likely to play an important role in this "developmental origins of health and diseases (DOHaD)" hypothesis (Waterland and Michels, 2007). DNA methylation is one of the pivotal mechanisms for the epigenetic regulation of gene transcription. The disruption of the DNA methylation pattern by prenatal chemical exposure is suspected to affect the development of offspring because the correct construction of this pattern is crucial for normal development. Several reports have suggested a relationship between developmental defects and the disruption of DNA methylation. Vinclozolin, an endocrine disruptor, which has antiandrogenic activity, transgenerationally alters the DNA methylation pattern in the male germ line (Anway *et al.*, 2005). It has been indicated that the alteration of DNA methylation induced by maternal ethanol consumption is capable of inducing changes in gene

Table 1. Characteristics of diesel exhaust exposure.

	CO (ppm)	SO_2 (ppb)	NO (ppm)	NO_2 (ppm)	NO_x (ppm)	DEP (μg/m^3)	DEP (number/cm^3)
Control air	0.504 ± 0.159	1.22 ± 0.83	0.003 ± 0.003	0.019 ± 0.008	0.022 ± 0.010	5 ± 4	4 ± 2
DE	1.84 ± 0.42	3.52 ± 1.20	0.456 ± 0.136	0.247 ± 0.070	0.703 ± 0.203	98 ± 29	70920 ± 24247

Values are the average concentration of each component in diesel exhaust and control air expressed as mean ± S.D.

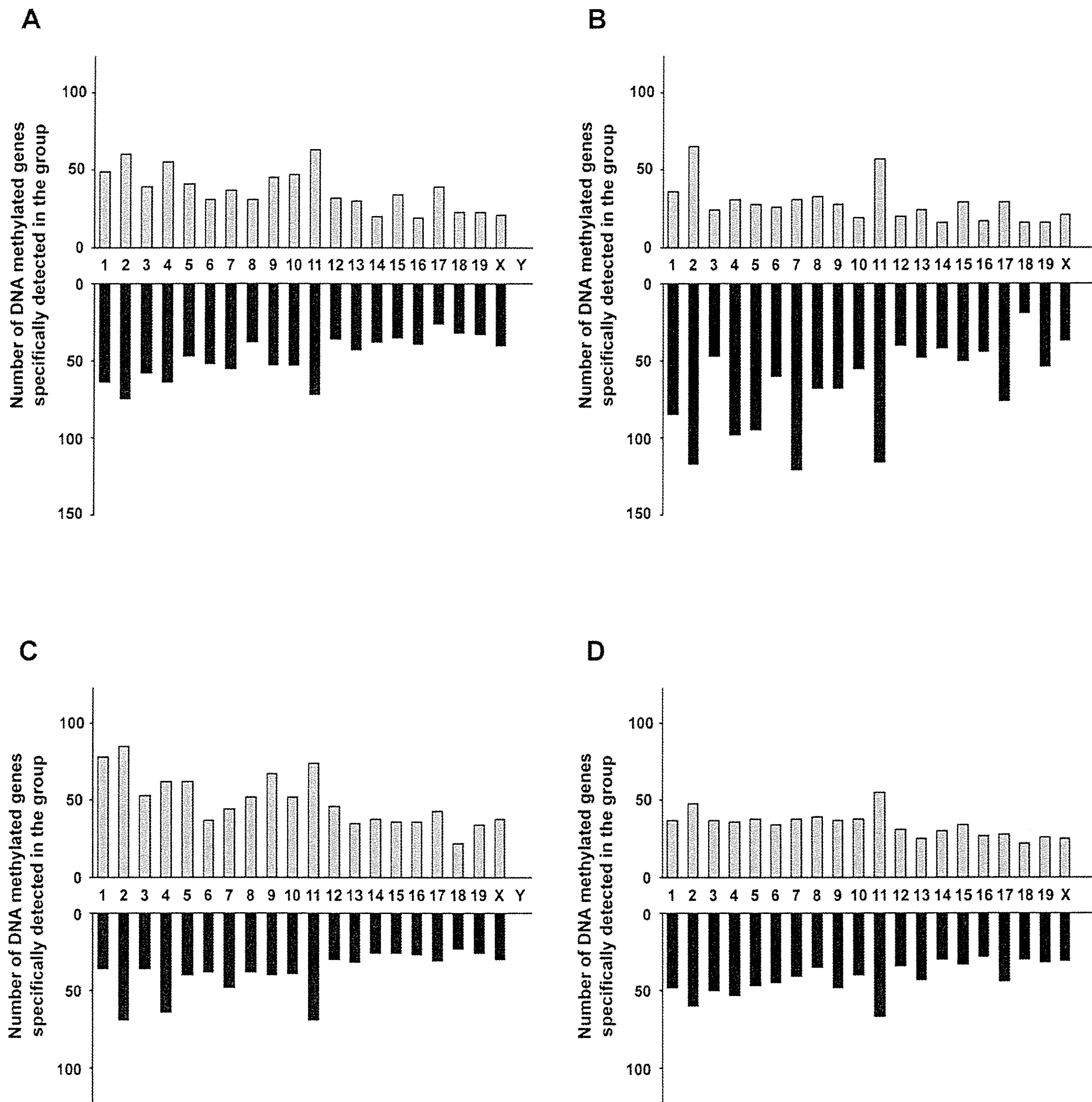

Fig. 1. Effects of prenatal diesel exhaust (DE) exposure on the DNA methylation state of gene promoters with CpG islands. The number of DNA methylated genes specifically detected in control and DE groups in 1-day-old (1d) male (A), 1d female (B), 21d male (C) and 21d female (D) offspring. The x-axis shows chromosome numbers. Black bars indicate the genes specifically methylated in control (meaning that DNA methylation was decreased by DE exposure). Gray bars indicate the genes specifically methylated in DE (meaning that DNA methylation was increased by DE exposure).

expression (Kaminen-Ahola *et al.*, 2010). At the time of writing, however, it remains unclear whether prenatal DE exposure affects the genome-wide DNA methylation pattern in the brain of offspring.

We produced a mass concentration of DEP at about 100 μg/m³, which is environmentally relevant. Previous report suggested that the effects of suspended PM in air pollutants are mainly derived from DEP (Donaldson *et al.*, 2005). Numerous megacities in the world demonstrate PM concentrations of 30-600 μg/m³ in annual averag-

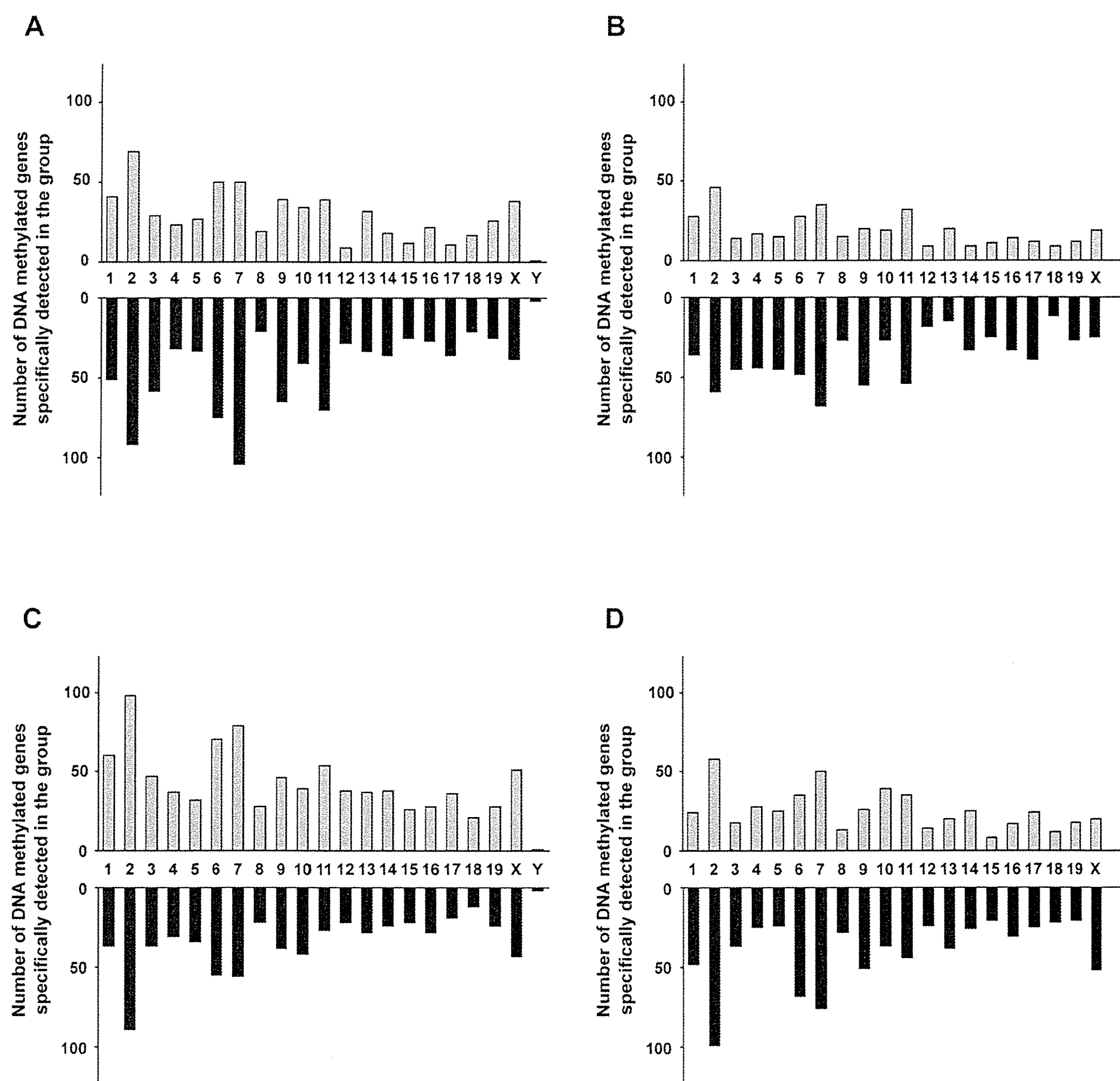

Fig. 2. Effects of prenatal diesel exhaust (DE) exposure on the DNA methylation state of gene promoters without CpG islands. The number of genes DNA methylated which were specifically detected in control and DE groups in 1-day-old (1d) male (A), 1d female (B), 21d male (C) and 21d female (D) offspring. The x-axis shows chromosome numbers. Black bars indicate the genes specifically methylated in control (meaning that DNA methylation was decreased by DE exposure). Gray bars indicate the genes specifically methylated in DE (meaning that DNA methylation was increased by DE exposure).

es (Gurjar *et al.*, 2008). For example, under the condition in the Mexico City (201 μg/m³) and assuming a ventilation rate of 6.4 L/min (9.2 m³/day) for a healthy adult at rest (Crosfill and Widdicombe, 1961), the total amount of PM exposure would be approximately 1,800 μg/m³. This would correspond to 6 μg/day of PM exposure for a mouse with an average ventilation rate of 0.021 L/min (Crosfill and Widdicombe, 1961). The DE exposure under the condition of present study was approximately 1 μg/day. In the present study, the DE exposure condition for DEP mass concentration and exposure time was designed to be lower than recent studies on the effects of DE exposure on the central nervous system (Levesque *et al.*, 2011a, 2011b; Win-Shwe *et al.*, 2012; Yamagishi *et*

Table 2. GO terms commonly enriched in male (M) and female (F) in 1-day-old offspring.

GO term	Gender	Enrichment factor	p-value
Positive regulation of neuron differentiation	M	2.01	0.007
	F	2.23	< 0.001
Ventricular septum morphogenesis	M	4.20	< 0.001
	F	3.39	< 0.001
Outflow tract morphogenesis	M	2.80	0.001
	F	2.69	< 0.001
RNA polymerase II core promoter proximal region sequence-specific DNA binding	M	2.17	0.025
	F	2.80	< 0.001
Embryonic cranial skeleton morphogenesis	M	2.56	0.015
	F	2.92	0.002
Histone deacetylase binding	M	2.10	0.008
	F	2.01	0.004
Cardiac muscle cell differentiation	M	2.19	0.048
	F	2.59	0.008
tRNA binding	M	2.47	0.032
	F	2.49	0.016

The enrichment factor for each category was defined as described in MATERIALS AND METHODS. Statistical analysis was performed using Fisher's exact test with hypergeometric distribution and the level of statistical significance was set at $p < 0.05$

Table 3. GO terms commonly enriched in male (M) and female (F) in 21-day-old offspring.

GO categories	Gender	Enrichment factor	p-value
Neurogenesis (positive regulation of neurogenesis)	M	2.83	0.008
	F	2.54	0.025
Hindbrain development	M	2.85	0.013
	F	4.93	< 0.001
mRNA transport	M	2.06	0.012
	F	2.77	< 0.001
Dorsal/ventral pattern formation	M	2.08	0.033
	F	2.66	0.003
Spermatid development	M	2.20	0.009
	F	2.33	0.006
Centriole	M	2.51	0.001
	F	2.28	0.007
Transcription cofactor activity	M	2.69	0.006
	F	2.72	0.011
Epithelial to mesenchymal transition	M	2.69	0.011
	F	2.66	0.020
Odontogenesis of dentin-containing tooth	M	2.02	0.026
	F	2.10	0.034
Protein targeting	M	2.26	0.028
	F	2.24	0.045

The enrichment factor for each category was defined as described in MATERIALS AND METHODS. Statistical analysis was performed using Fisher's exact test with hypergeometric distribution and the level of statistical significance was set at $p < 0.05$.

al., 2012).

In the present study, we used the combination of MeDIP and subsequent promoter tilling array analysis to examine the effects of prenatal DE exposure on the genome-wide promoter DNA methylation state in the brain of offspring mice. Our results revealed that prenatal DE exposure disrupted the DNA methylation state of offspring in all chromosomes rather than a particular

chromosome. Furthermore, altered DNA methylation was observed in both 1d and 21d offspring. To understand the molecular events influenced by DE exposure, differentially methylated genes were bioinformatically categorized using GO terms. This bioinformatic interpretation indicated that differentially DNA methylated genes were enriched in the GO terms related to neuronal differentiation and neurogenesis. These results suggest that aberrant DNA methylation induced by prenatal DE exposure affects neuronal development. The fetal and neonatal period is critical for the development and organization of the neuronal network (Sporns *et al.*, 2004; Smyser *et al.*, 2010). We previously reported that prenatal DE exposure affects spontaneous locomotor activity and monoaminergic system in mice (Suzuki *et al.*, 2010). The genes which aberrant DNA methylation was observed in this study would be associated with development and organization of the monoaminergic system in mice. The detailed analysis about this point is required to clarify the association between aberrant DNA methylation and functional changes in mice.

The regulation of gene expression during fetal and neonatal period is associated with morphological and functional development of the brain (Muotri and Gage, 2006). Given that the established DNA methylation pattern is generally maintained through cell division (Bergman and Cedar, 2013), it is predicted that altered DNA methylation would be partially maintained after development. Several reports suggest a relationship between aberrant DNA methylation and neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's disease (Jakovcevski and Akbarian, 2012). In addition, cortical neuron degeneration has also been observed in canines that inhaled air pollutants containing PM (Calderón-Garcidueñas *et al.*, 2002). When the information from these reports is considered, it would seem that altered DNA methylation induced by prenatal DE exposure would also be associated with the later pathogenesis of neurodegenerative disorder. In the present study, we observed that the genes which showed altered DNA methylation were different between 1d and 21d offspring. These results suggested that the aberrant DNA methylation pattern induced by prenatal DE exposure was partially recovered during growth. Further examinations about DNA methylation in young adult mice are required to clarify the DNA region which shows persistent aberrant DNA methylation.

The authors detected the altered DNA methylation of the genes independently of the presence of CpG islands. Several reports indicate a relationship between reactive oxygen species (ROS) and DNA methylation. Oxidative DNA damage is known to disturb the binding of methyltransferase to the DNA (Valinluck *et al.*, 2004), thus resulting in hypomethylation of cytosine residue. Weitzman *et al.* (1994), showed that DNA methylation can be influenced by free radical adducts on adjacent guanine residues. Taken together, it seems possible that ROS associated with DE inhalation (Li *et al.*, 2010) could disrupt the DNA methylation state in the developing tissues. Since DNA hypomethylation induces genomic instability (Chen *et al.*, 1998), a decrease in genome-wide DNA methylation may lead to an increase in the mutation rate that is induced by prenatal DEP exposure (Ritz *et al.*, 2011).

We previously reported a bioinformatic method for locating candidate brain regions of interest for the effects of nanoparticle exposure using MeSH terms (Umezawa *et al.*, 2012). We applied the method to survey the brain regions that are preferentially affected by prenatal DE exposure. Although several MeSH terms related to brain region were enriched in each experimental group, no common regions were found in the comparisons. With regard to the effects of DE exposure, it therefore seems less likely that any brain region is a specific target for DNA methylation disruption.

In the present study, we interpreted the biological effects caused by differential gene methylation using a bioinformatic method. Further "wet experiments" are required to clarify whether disrupted DNA methylation actually alters the gene expression, neural differentiation, and the function of central nervous systems especially monoaminergic systems which are affected by prenatal DE exposure (Suzuki *et al.*, 2010). Additionally, the molecular mechanisms underlying the effect of prenatal DE exposure on the DNA methylation pattern remain unknown. As indicated above, the disturbance of DNA methyltransferase binding (Valinluck *et al.*, 2004) is potentially involved in the dysregulation of DNA methylation. In addition, the biological systems that determine the DNA regions that are methylated are another possible target of DE exposure. Previous reports indicated some factors essential for the establishment and maintenance of the methylation imprint, including Zfp57 and PGC7/Stella (Li *et al.*, 2008; Nakamura *et al.*, 2007). Shen *et al.* (2013), showed that a dynamic methylation-demethylation cycle occurs at a large number of genomic loci. These molecules and pathways would also be candidate targets of prenatal DE exposure. Recently, a portion of the piRNA, small RNA exclusively expressed in the germ line, was linked to *de novo* DNA methylation (Olovnikov *et al.*, 2012). Wick *et al.* (2010) showed that particles up to a diameter of 240 nm were taken up by

the placenta and, further, were able to cross the placental barrier. The findings in this report suggest that a part of DEP, especially nano-sized particles (diameter < 100 nm), might be transferred to fetus. On the other hand, Weaver *et al.* (2005) showed maternal stress alters the epigenotype in rodent offspring. The analysis about whether DEP or maternal stress disrupts the molecules/pathways which indicated above would help to solve the problem.

Our results showed that altered DNA methylation pattern was different between male and female offspring. Previous reports suggest that steroid hormone and endocrine disruptor change DNA methylation (Jost and Saluz, 1993; Anway *et al.*, 2005; Skinner *et al.*, 2010). Watanabe and Kurita (2001) showed the possibility that prenatal DE exposure alters fetal testosterone levels. Brain sex differences organized by a transient hormone surge may be maintained through epigenetic modification (McCarthy *et al.*, 2009). Our results, combined with these reports, showed the possibility that prenatal DE exposure affects the brain sex difference through alteration of DNA methylation in the developmental stage.

In conclusion, the present study showed that prenatal DE exposure disrupts the genome-wide DNA methylation state in the brain of offspring mice. Bioinformatic GO analysis showed that differentially DNA methylated genes were enriched in neuronal differentiation. These results suggest that disrupted DNA methylation in the infertile mouse brain is involved in neural dysfunctions induced by prenatal DE exposure. Bioinformatic interpretation of the altered DNA methylation data using GO terms may provide clues that lead to the better understanding of the molecular events underlying the effects of prenatal DE exposure in the developmental period. In addition, a decrease in genome-wide DNA methylation may lead to increased mutation rate, which is induced by prenatal DEP exposure. Our results suggest that the early-life social environments in which DE is present could be critical for the construction of the DNA methylation pattern and may be associated with a long-term impact on health.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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