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4　ナノマテリアルの次世代影響—脳神経系及び雄性生殖系を中心に

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4.1　はじめに

筆者らは，ディーゼル排ガスの胎仔期曝露の系において，排ガス由来と思われる超微小粒子（ナノ粒子）が仔の脳の特定な細胞の特定なオルガネラに蓄積され，周辺の細胞に影響を及ぼしている像を得た[1]。母マウスから胎盤を介して胎仔脳に移行，取り込まれ，出生後も排出されず留まったものと予想した。しかし，それらが排ガス由来のナノ粒子であるという絶対的な証拠を得ることは困難であった。そこで，金属ナノ粒子に注目し，化粧品や光触媒として汎用される酸化チタンナノ粒子が妊娠期のマウスから仔の脳や精巣に移行し，様々な異常を生じさせることを明らかにした[2]。移行した粒子状物質が酸化チタンであることの証拠を得た。他のナノマテリアルに関しても研究を進め，次世代に様々な健康影響を及ぼすことを明らかにしつつあるが，本稿では酸化チタンナノ粒子の健康影響を中心に，我々が得た次世代の脳神経系及び生殖系に及ぼす影響に関する結果を話題として紹介したい。

4.2　酸化チタン（TiO_2）ナノ粒子とは？

TiO_2にはアナターゼ型，ルチル型，ブルッカイト型の主に3種類の結晶構造が存在する。最も広く用いられているアナターゼ型のTiO_2は光触媒作用が強く，このとき産生されるスーパーオキサイドや過酸化水素，ヒドロキシラジカルなど活性酸素が細胞毒性を示すことが報告されている[3,4]。この作用を利用して環境浄化や殺菌，抗菌などの目的でナノ粒子が実用化されている。ルチル型TiO_2は，これまで安全だと考えられてきたため，化粧品の他に医薬品や食品添加物としても利用されている。白色顔料，食品着色剤の成分であり，大きなサイズの粒子では細胞に不活性，あるいは人体にほとんど影響がないことが確認され，汎用されてきた。

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また，ナノサイズ化することにより紫外線分散作用を獲得し，受けた光の反射の度合いを低下させるために仕上がりが白くならずに透明度が増すこと，また粒子サイズが小さいためにさらっとした塗り心地になることなどから，日焼け止めやファンデーションに利用されている。

4.3　酸化チタンナノ粒子の取り込み

これまでの研究において，TiO_2が皮膚を透過することを明確に示す報告はなく，皮膚の表面や角質層に留まるとされている[5~7]。しかし，化粧品は長年にわたって人体に直接繰り返し塗布されること，粒子のサイズがナノスケールであること，表面の物理化学的性質により皮膚のバリア機構に対する透過性が変わることなどから，血液やリンパ液を介して全身を巡り，蓄積され，影響を及ぼす可能性が残る。また，基材や界面活性剤等の添加物との組み合わせにより粒子の挙動が変化する可能性もある。さらに，アトピー性皮膚炎や紫外線照射などにより皮膚が傷ついている場合や炎症を起こしている場合には角質層の透過性が増大していることが報告されている[8,9]。そこで，今後できるだけ多くの可能性を検証しておく必要性がある。一方，酸化チタンナノ粒子が作業環境中あるいはその他の環境下において浮遊する場合は，呼気から取り込まれる可能性があり，呼吸器からの取り込みの影響を確認することも重要である。

4.4　成獣における酸化チタンの脳への移行

ナノマテリアルの脳への移行についてOberdörsterらは，ラットの吸入実験において，鼻部上皮に沈着した^{13}Cナノ粒子が，嗅覚神経を介して，脳へアクセスすることを実証した10)。一方，Wangらは，マウス成獣の鼻腔に酸化チタン(粒径80nmルチル型，粒径155nmアナターゼ型)を大量に添付すると嗅球を介して脳全体に移行すること，特に海馬に蓄積することを観察した[11]。いずれも，ナノ粒子は鼻腔から嗅覚神経の軸索輸送で脳内に僅かに移行したと考えられている。

Fabianらは，ラットに酸化チタンナノ粒子(粒径20～30 nm)を静脈投与し，酸化チタンが肝臓，肺，脾臓，腎臓の順に高濃度に検出されること，脳，リンパ液には検出されないことを報告している。肺と腎臓は14日間でコントロールレベルに濃度が低下したが，肝臓は28日後も高レベルを維持し，脾臓はわずかに減少した[12]。Sugibayashiらはマウスに酸化チタンナノ粒子(粒径15 nm)を静脈投与し，投与後5分で血液や各種臓器において濃度が上昇したが，脳には検出されなかったと報告している。肝臓では，1ヶ月後でも30%しか減少していない[13]。

以上，成獣では脳血液関門があり，血液を介しては脳には検出できるほどの酸化チタンは移行しないものと考えられている。

4.5　酸化チタンナノ粒子の妊娠期母獣から仔への移行

筆者らは，酸化チタンナノ粒子が妊娠期の母から仔に移行し，出生後の成長期にも脳内に取り込まれた状態で残り，病理学的に，また，機能的に様々な影響を及ぼすことを世界に先駆けて明らかにした[2]。

投与条件：妊娠ICR系マウスを使用した。アナターゼ型のTiO_2(Sigma-Aldrich, Saint Louis, MO, USA；粒子径25～70 nm)を1 mg/mLとなるように調製し，ソニケーターで撹拌して分散させた。溶媒は，生理食塩水に分散媒としてTween-80を0.05 %となるように加えて調製した。この原液を10分の1ずつ1000分の1まで段階希釈した。調製したTiO_2分散液を妊娠6, 9, 12, 15日目に各々0.1 mL(TiO_2投与量100 μg/mL)皮下投与した。対照群には溶媒を同量皮下投与した。

酸化チタンの検出：妊娠期に投与したナノ粒子が，産仔に移行するかを検討した。血液脳関門，精巣関門などバリアー機能が未発達な胎仔期にナノ粒子を曝露すると，ナノ粒子が胎盤を経由して母獣から仔に移行し，さらに脳や精巣へ移行すること，それぞれの機能に影響を及ぼすことを示唆する結果を得た。妊娠期のマウスに酸化チタンナノ粒子を皮下投与し，産仔6週齢時に精巣組織の透過型電子顕微鏡(TEM)観察および走査型電子顕微鏡–X線スペクトロ測定装置(FE-SEM/EDS)による解析を行ったところ，脳(大脳皮質，海馬，嗅球など)や精巣の細胞(ライディヒ，セルトリ，精子細胞など)に酸化チタン粒子が検出・同定された(図1，図2，図3)。

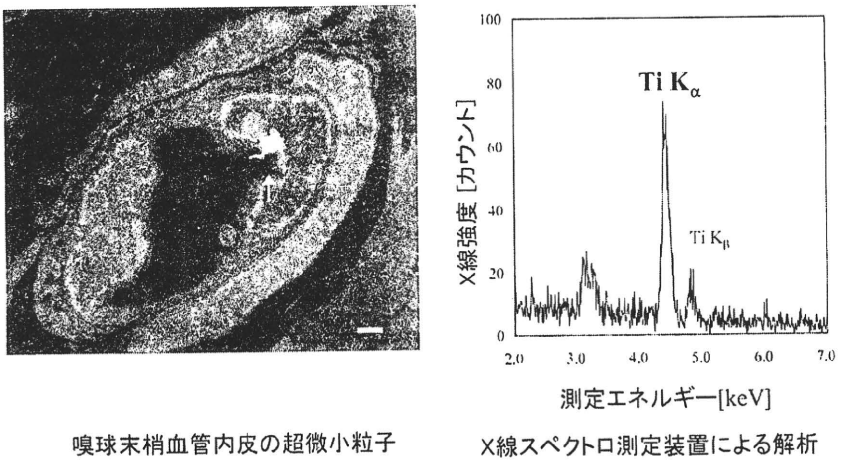

図1　酸化チタンナノ粒子の産仔への移行

胎仔期に酸化チタンナノ粒子の曝露を受け，成長した仔(6週)の脳の嗅球末梢血管内皮に取り込まれている粒子の解析(スケールバーは1 μm)。X線スペクトロ解析により，粒子が酸化チタンであることが同定された。(解析は鈴木健一郎が行った。文献2参照)

4.6　酸化チタンナノ粒子の胎仔期曝露による脳神経系への影響

ナノ粒子曝露群の脳の各部位について病理組織学的検討を行ったところ，6週齢雄の脳末梢血管に多発性の微小梗塞の所見が認められ，嗅球の僧帽細胞がカスパーゼ-3(アポトーシスのマーカー)陽性細胞になっていることが認められた。この結果，TiO_2 を妊娠マウスに投与すると TiO_2 は胎盤を経由して胎仔に移行し，未発達な血液脳関門を通過し，脳各部位に残ること，脳の機能に影響を及ぼすことが示唆された[2]。

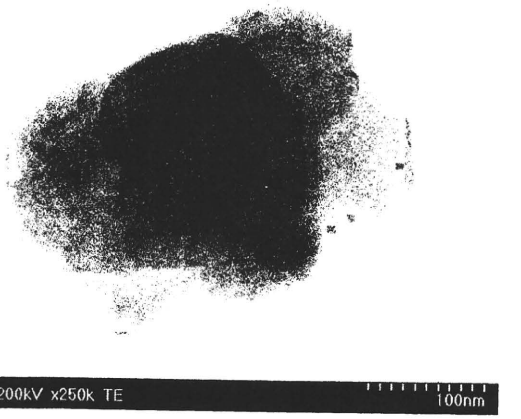

図2　大脳皮質神経細胞に取り込まれた酸化チタンナノ粒子の拡大電子顕微鏡写真(複数の粒子が凝集している[2])

4.7　酸化チタンナノ粒子の胎仔期曝露による雄性生殖系への影響

酸化チタンナノ粒子曝露群の産仔6週齢の精巣組織染色の観察では精細管を構成する細胞に異常が認められた。また，セルトリ細胞の電子顕微鏡観察ではミトコンドリアの膨潤化，クリステの消失が認められ，1日精子産生数，セルトリ細胞数の有意な低下が認められた[2]。さらにその後の研究において，酸化チタンはアナターゼ型以外にも化粧品素材として汎用されるルチル型及びその表面加工粒子においても雄性生殖系に対する影響が認められた。それらの影響の一部は1000分の1に希釈しても認められた。

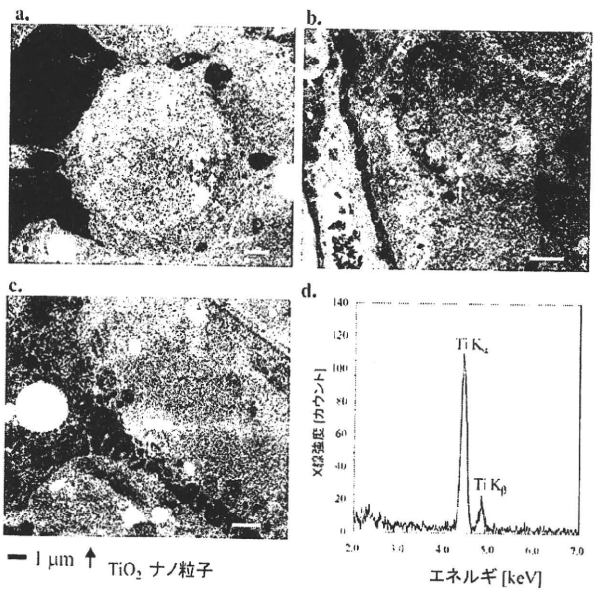

図3　精巣の細胞に取り込まれた粒子の酸化チタンナノ粒子の電子顕微鏡写真とX線スペクトロ解析
(a. ライディヒ細胞，b. セルトリ細胞，c. 精子細胞)

4.8　考察

筆者らは動物実験を通して以

下の結論を得た。光触媒や化粧品に汎用される酸化チタンナノ粒子は，動物体内に入ると，血流にのり，全身の組織・臓器を駆け巡る。また，妊娠した母から胎盤を介して仔に移行し，未発達な脳血液関門，精巣血液関門などを通過し，周辺の細胞に影響を及ぼしうる(図4)。吸入，気管内投与，点鼻投与，皮下投与など投与法に関わらずナノマテリアルが妊娠した母マウスの血流にのれば仔に移行し，影響を及ぼす。生まれてから成長する過程で様々な症状として現れることがあり，それらは時として，重大な疾患の発症，増悪化に繋がる恐れがある。皮膚に塗布された化粧品中のナノ粒子や作業現場等で浮遊するナノ粒子がどのような機構でどの程度血中に移行するのか，詳細に検討する必要があり，今後の課題である。

英国の David Barker 博士は疫学調査の結果に基づいて 1986 年に「成人病胎児期起源説」を唱えている[14]。胎児期における栄養不足は，臓器の十分な発育を妨げる要因になっているが，それらを補う体内システムの形成が生活習慣病とよばれている成人病の発症に繋がるという仮説である。胎児期や乳幼児期にその素因が形成され，出生後の環境要因によって成人病などの疾病に罹りやすくなる。胎仔期に酸化チタンなどナノ粒子の曝露を受けると，出生後の発達に応じて脳神経系をはじめ生殖系やその他の臓器で様々な症状が現れるという我々の研究結果は，彼の仮説を支持している[15,16]。次世代の仔には僅かなナノ粒子しか移行していないはずだが，何故極微量のナノマテリアルがこのような大きな影響を長期間にわたって及ぼすのか，謎である。その分子機構の解明が待たれる。

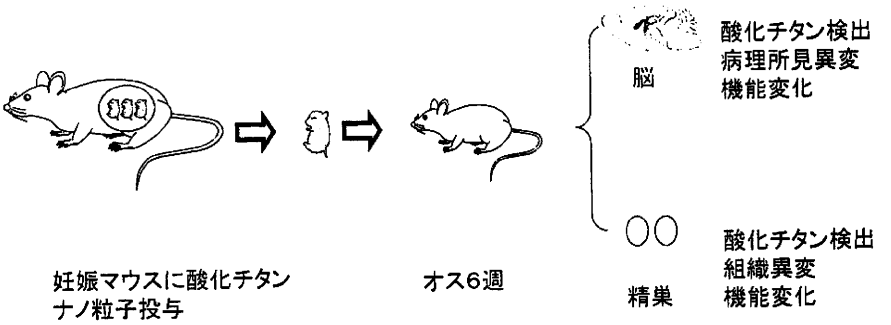

図4　酸化チタンナノ粒子の次世代への影響

酸化チタンナノ粒子を妊娠期のマウスに曝露すると，成長後の仔の脳や精巣に粒子が検出され，様々な影響が認められる。(文献2の内容を要約した模式図)

4.9　おわりに

ナノマテリアルの有害性と健康への影響の実態が明らかになれば，予防対策は立てやすくなる。さらには治療法も考えられるようになる。一方では，ナノテクノロジーは科学技術基本計画や新産業創造戦略において，推進すべき重要な政策として位置づけられており，産業発展の

ため必須の科学技術である。従って,わが国が産業立国として,21世紀の新たな産業技術をリードしていくためにもその基盤となるナノマテリアルの健康への影響，特に次世代への健康影響を明確にして，十分な対策を構築することが極めて重要な課題である。

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Letter

Prenatal exposure to titanium dioxide nanoparticles increases dopamine levels in the prefrontal cortex and neostriatum of mice

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ABSTRACT — Titanium dioxide (TiO_2) nanoparticles are widely used in cosmetics, sunscreen and as a photocatalyst. However, little is known about the biological effect of TiO_2 nanoparticles in humans and other animals. Here, we investigated whether prenatal exposure to TiO_2 nanoparticles impacted the central nervous system in mice. We measured the levels of dopamine (DA) and its metabolites in several regions of the brain in mice using high performance liquid chromatography (HPLC). HPLC analysis showed that DA and its metabolites were increased in the prefrontal cortex and the neostriatum following prenatal exposure to TiO_2 nanoparticles. The present study highlights the possibility that maternal exposure to TiO_2 nanoparticles might influence the development of the central dopaminergic system in offspring.

Key words: Brain, Dopamine system, Nanoparticle, Prenatal exposure, Titanium dioxide (TiO_2)

INTRODUCTION

Nanocrystalline titanium dioxide (TiO_2), a noncombustible, odorless powder, is an important material used in commerce and can be found in paints, cosmetics, food additives and implanted biomaterials (Kaida *et al.*, 2004; Esterkin *et al.*, 2005).

TiO_2 nanoparticles have three structural isoforms, anatase, rutile and brookite. Since the photocatalytic activity of the anatase form of TiO_2 is higher than the rutile form (Sayes *et al.*, 2006), anatase TiO_2 is currently used in products as diverse as sterilization materials and coatings for self-cleaning windows and walls (Fujishima *et al.*, 2008). Although TiO_2 was thought to be a non-toxic material, several studies have suggested that TiO_2 nanoparticles may be toxic to living systems.

Fabian *et al.* (2008) demonstrated that, following intravenous injection of TiO_2 nanoparticles into living animals, the particles enter the systemic circulation and migrate to various organs and tissues. There may be a critical size beyond which movement of the nanoparticles within the body is restricted. Oberdörster *et al.* (2004) reported that inhaled nanoparticles could enter the brain *via* the olfactory nerves. Wang *et al.* (2008a) demonstrated that intranasally instilled TiO_2 nanoparticles could be translocated into the central nervous system of mice *via* the olfactory nerve tract, and accumulated in the olfactory nerve layer, cerebral cortex, thalamus and hippocampus. The oxidative damage expressed as lipid peroxidation increased significantly. Exposure to anatase TiO_2 particles also produced significant inflammation (Wang *et al.*, 2008b).

However, the potential toxicity of TiO_2 in the next generation has yet to be examined. We have already demonstrated that prenatal exposure to diesel exhaust (DE) affects the dopaminergic system resulting in a reduction in locomotion in mice (Yokota *et al.*, 2009). Moreover, Sugamata *et al.* (2006) found that granular perithe-

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lial cells, scavenger cells surrounding cerebral vessels, showed signs of apoptosis; the cytoplasmic granules had degenerated and showed evidence of what appeared to be ultrafine, DE particles in the brain of offspring exposed to DE during the fetal period. These results suggest that the nanoparticles in DE might enter the fetal circulation *via* the placenta and affect the central nervous system.

We have demonstrated that nano-sized TiO_2, administered subcutaneously to pregnant mice, was transferred to the offspring and affected the genital and cranial nerve systems of male offspring. Nanoparticles identified as TiO_2 by energy-dispersive X-ray spectroscopy were found in the testes and brain of exposed 6-week-old male mice. In the offspring of TiO_2-injected mice, various functional and pathologic disorders were observed (Takeda *et al.*, 2009). Recently, we have also reported that maternal exposure of mice to TiO_2 nanoparticles may affect the expression of genes related to the development and function of the central nervous system (Shimizu *et al.*, 2009).

In the present study, we investigated the impact of prenatal exposure to TiO_2 nanoparticles on the dopaminergic system. We measured the levels of dopamine (DA) and its metabolites in several regions of the brain in mice using high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

TiO_2 nanoparticles

TiO_2 nanoparticles (anatase form, particle size 25-70 nm, surface area 20-25 m^2/g) were purchased from Sigma-Aldrich (St Louis, MO, USA) and diffused in saline containing 0.05% Tween 80. The sample solution was sonicated for more than 30 min immediately before administration (28 kHz, 60 w; Sonicator, Medical Aiwa Co., Ltd., Tokyo, Japan). The distribution of TiO_2 particles of different diameters was determined by field emission-type scanning electron microscopy (FE-SEM). Diameter of the particles was measured on a randomly selected area of the FE-SEM image. A wide distribution of single TiO_2 powder size was confirmed which ranged from 20 to 100 nm. The size distribution of the TiO_2 nanoparticles in the suspension was measured by dynamic light scattering (DLS) using FPAR-1000 (Otsuka Electronics Co., Ltd., Osaka, Japan), and the agglomeration state was assessed by transmission electron microscopy (TEM) (JEM-1200 EXII, JEOL, Ltd., Tokyo, Japan). The size distribution was determined with the algorithm CONTIN. For the TEM assessment, an aliquot of 5 µl was placed on the copper grid coated with hydrophilized formbar and assessed with an accelerating voltage of 80 kV. Zeta potential of TiO_2 in this condition was negative (Bihari *et al.*, 2008).

Animals

Pregnant ICR mice were purchased from the SLC Co. (Shizuoka, Japan). TiO_2 nanoparticles were suspended at 1 mg/ml, and 0.1 ml was administered subcutaneously to the pregnant ICR mice at gestation days 6, 9, 12, 15 and 18. Control animals were treated with saline containing 0.05% Tween 80. In each group, pups were weaned on postnatal day 21. They were maintained in a temperature- and light-controlled environment with free access to standard rodent food and water. All experimental animals were handled in accordance with the institutional and national guidelines for the care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering.

Neurochemical analysis

Brains were obtained from 6-week-old anesthetized male pups after decapitation and dissected into ten regions: olfactory bulb, prefrontal cortex, neostriatum, nucleus accumbens, hippocampus, amygdala, hypothalamus, midbrain (containing the ventral tegmental area and substantia nigra), brainstem (containing the raphe nucleus and locus coeruleus) and cerebellum. The dissected regions were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Frozen brain tissues were homogenized in ice-cold 0.2 M perchloric acid containing 100 mM EDTA (2Na) and 100 ng isoproterenol, as an internal standard. The homogenates were centrifuged at 20,000 x *g* for 15 min at 0°C. Supernatants were transferred to new tubes and the pellets were stored for protein assay. The pH of the supernatant was adjusted to 3.5 with 1 M sodium acetate, and stored at -80°C until analysis. For HPLC, 10 µl of the pH-adjusted supernatant were injected into an HPLC system with electrochemical detection (EICOM Co., Kyoto, Japan). DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine hydrochloride (3-MT) were separated by a C18 reverse-phase column (EICOM, EICOMPAK SC-5ODS, EICOM Co.) with a mobile phase containing 0.1 M sodium acetate, citric acid monohydrate, EDTA (2Na) (5 mg/l), sodium 1-octanesulfonate (190 mg/l), and 15% methanol.

Protein assay

Pellets were resuspended in 100 mM Tris-HCl for protein determination by the high-sensitivity Bradford method using a commercial reagent (ADV-01, Cytoskeleton, Inc., Denver, CO, USA). Measurements were performed according to the manufacturer's protocol.

Statistical analysis

Data are expressed as mean ± S.E.M. Differences between groups were examined for statistical significance using a Mann-Whitney *U*-test and $p < 0.05$ indicated statistical significance.

RESULTS

Size distribution and agglomeration state in suspensions of TiO_2 nanomicroparticles

A TEM image of the state of TiO_2 nanoparticles dispersed in saline containing 0.05% Tween 80 is shown in Fig. 1a. TiO_2 nanoparticles were easily aggregated, and the majority of particles were agglomerated. The size distribution of TiO_2 nanoparticles in the suspension was analyzed by DLS. TiO_2 demonstrated a wide range in size distribution from 20 to 12,805 nm, and the most abundant sizes were two peaks at 27 ± 4 and 2,429 ± 1,906 nm, respectively (Fig. 1b).

Monoamine levels in 10 regions of the brain

Monoamine levels were determined in 10 regions of the brain: the olfactory bulb, prefrontal cortex, neostriatum, nucleus accumbens, hippocampus, amygdala, hypothalamus, midbrain, brainstem and cerebellum. In the prefrontal cortex, DA and its metabolites (DOPAC, HVA, 3-MT) were increased in TiO_2 nanoparticle-exposed mice (DA, + 109%; DOPAC, + 46%; HVA, + 48%; 3-MT, + 56%; Fig. 2a) over control levels. In the neostriatum, DA and metabolites (DOPAC, HVA) were increased (DA, + 39%; DOPAC, + 43%, HVA, + 45%; Fig. 2b). In the other regions of the brain, the levels of DA and its metabolites were not altered significantly (Table 1).

DISCUSSION

The purpose of this study was to determine the effect of prenatal exposure to TiO_2 nanoparticles on the dopaminergic system of the developing mouse brain. The levels of DA and its metabolites were determined individually using HPLC. Significant increases in the amount of DA and DA metabolites were observed in the striatal and prefrontal area of the TiO_2-exposed group compared to the control animals (Fig. 3). We have already reported that exposure to DE during embryonic development altered the level of DA in the nucleus accumbens, leading to alterations in the spontaneous motor activity of the offspring (Yokota *et al.*, 2009). Several reports demonstrated that increase in the levels of DA metabolite indicate the increase in the DA neurotransmission (Saraswat *et al.*, 1981; Narita *et al.*, 1993). Taken together, our findings suggest that prenatal exposure to nanoparticles may influence the dopaminergic system in the brain.

There are two major dopaminergic systems in the brain: the nigrostriatal pathway and the mesolimbic pathway (Hornykiewicz, 1971). The former connects the substantia nigra pars compacta to the striata and plays a role in the control of motor function (Andén *et al.*, 1966). The mes-

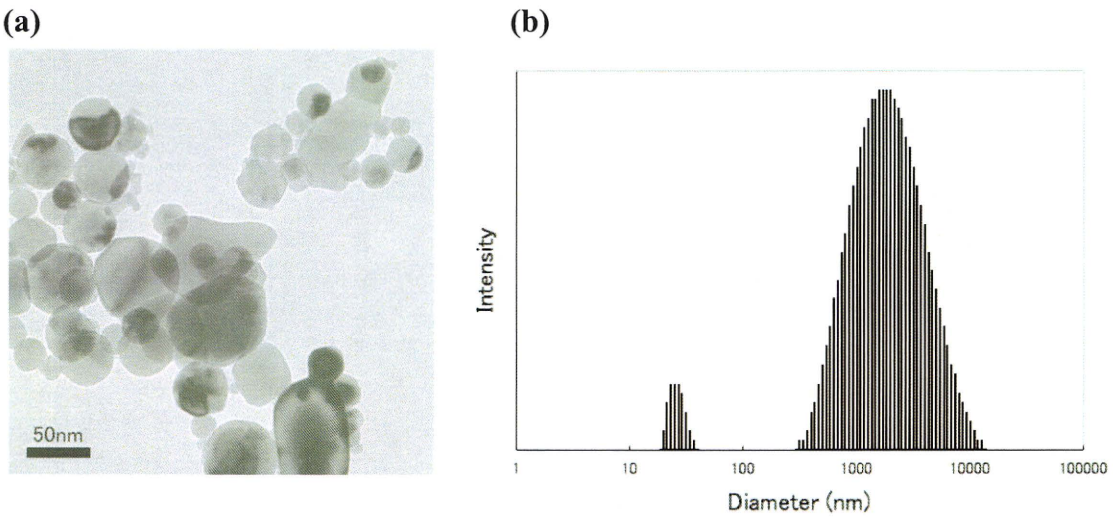

Fig. 1. TEM image of TiO_2 nanoparticles and their size distributions. TiO_2 nanopowder was suspended in saline with 0.05% (v/v) Tween 80 and sonicated for more than 30 min immediately before administration. (a) The agglomeration state was assessed by TEM and (b) the size distribution of the TiO_2 nanoparticles in the suspension was measured by DLS.

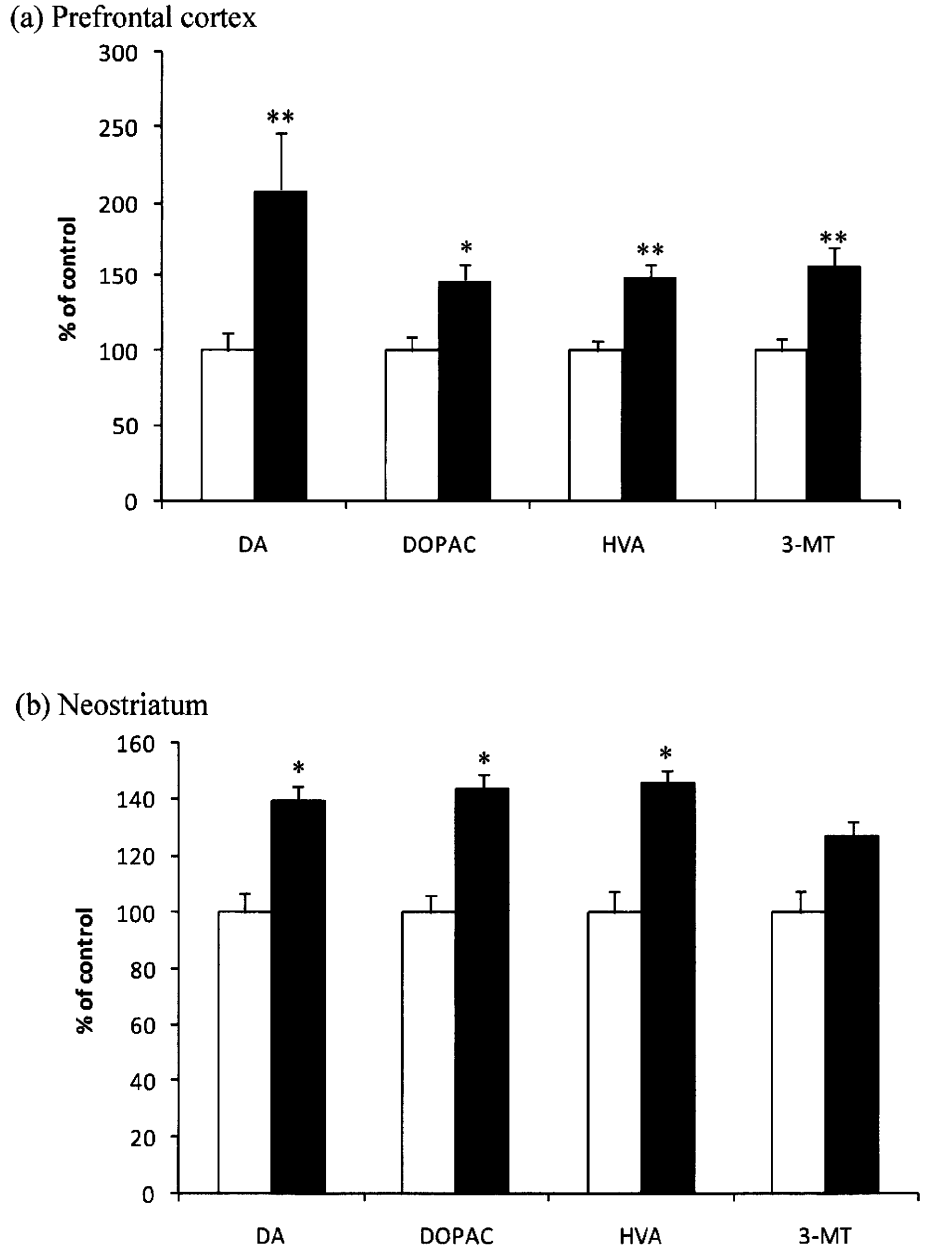

Fig. 2. Changes in the levels of DA and its metabolites (DOPC, HVA, 3-MT) in the (a) prefrontal cortex and (b) neostriatum obtained from 6-week-old male mice exposed, prenatally, to TiO_2 nanoparticles or from control animals. The data are expressed as a percentage of the value in control mice. Each column represents the mean ± S.E.M. (n = 8). *$p < 0.05$, ** $p < 0.01$ vs. each control group.

olimbic pathway extends from the ventral tegmental area to the nucleus accumbens, amygdala and prefrontal cortex. It plays a critical role in the control of cognitive and emotional function (Tzschentke, 2001; Alcaro *et al.*, 2007). It has been reported that abnormalities in the monoaminergic systems are associated with psychiatric diseases such as schizophrenia, depression, anxiety and attention-deficit hyperactivity disorder (ADHD) (Tamminga, 2006; Ressler and Nemeroff, 2000). Additionally, defects in the dopaminergic system are associated with psychiat-

Table 1. Effects of prenatal exposure to TiO_2 nanoparticles on the central dopaminergic system of offspring

Brain region	Group	Content (pg/mg protein)			
		DA	*DOPAC*	*HVA*	*3-MT*
Olfactory bulb	*Control*	4,682 ± 638	346 ± 44	1,615 ± 138	749 ± 24
	TiO_2	4,444 ± 272	346 ± 33	1,766 ± 140	719 ± 39
Prefrontal cortex	*Control*	3,121 ± 345	1,380 ± 135	3,608 ± 275	612 ± 44
	TiO_2	6,534 ± 1,123**	2,022 ± 181*	5,352 ± 342**	956 ± 78**
Neostriatum	*Control*	194,928 ± 16,063	25,293 ± 2,465	22,408 ± 2,256	22,165 ± 1,744
	TiO_2	270,793 ± 13,886*	36,239 ± 1,280*	32,510 ± 1,143*	28,015 ± 1,595
Nucleus accumbens	*Control*	196,751 ± 6,459	34,169 ± 2,495	24,305 ± 1,191	20,893 ± 1,249
	TiO_2	209,617 ± 6,956	34,420 ± 1,466	27,467 ± 710	19,717 ± 868
Hippocampus	*Control*	2,865 ± 1,408	1,002 ± 381	1,227 ± 364	1,433 ± 417
	TiO_2	1,471 ± 806	450 ± 111	1,105 ± 150	986 ± 214
Hypothalamus	*Control*	16,560 ± 955	6,462 ± 458	4,678 ± 351	2,486 ± 152
	TiO_2	15,759 ± 1,616	5,512 ± 184	4,191 ± 204	2,261 ± 117
Amygdala	*Control*	17,440 ± 1,786	3,960 ± 352	1,683 ± 181	3,490 ± 313
	TiO_2	14,475 ± 1,192	3,913 ± 252	1,676 ± 226	2,956 ± 229
Midbrain	*Control*	8,430 ± 1,056	5,185 ± 738	4,175 ± 586	1,302 ± 154
	TiO_2	9,163 ± 471	5,494 ± 274	4,964 ± 293	1,442 ± 118
Brainstem	*Control*	1,374 ± 63	1,237 ± 98	2,277 ± 127	198 ± 15
	TiO_2	1,655 ± 252	1,347 ± 232	2,744 ± 444	228 ± 20
Cerebellum	*Control*	N.D.	N.D.	576 ± 58	N.D.
	TiO_2	N.D.	N.D.	765 ± 59	N.D.

The suspended TiO_2 nanoparticles were administered subcutaneously to the pregnant ICR mice at gestation days 6, 9, 12, 15 and 18. Control animals were treated with vehicle (saline with 0.05% Tween 80). In each group, pups were weaned on postnatal day 21. Levels of DA and its metabolites (pg/mg protein) in each area of the brain. Data are presented as mean ± S.E.M. (n = 8). *$p < 0.05$, **$p < 0.01$ vs. each control group, N.D. - not detectable.

ric pathologies such as ADHD and schizophrenia (Thapar *et al.*, 2005; Wong *et al.*, 1986).

Psychiatric conditions are regarded as prenatal developmental disorders of the brain that are associated with heritable and environmental factors. Therefore, an increase in striatum levels of DA leads to enhancement of locomotor activity. Furthermore, it has been reported that the enhancement of DA metabolic turnover in the prefrontal cortex results in impairments of working spatial memory (Hirvonen *et al.*, 2005). Taken together, these findings suggest that the increase in DA observed in this study might affect motor and cognitive functions.

Many studies have shown that nanoparticles produce reactive oxygen species and cause oxidative damage to cells and tissues (Wang *et al.*, 2008a, 2008b; Hussain *et al.*, 2009). It is uncertain whether the changes in DA levels in response to prenatal exposure to TiO_2 nanoparticles resulted from an increase in the production of reactive oxygen species. Notably, we have also observed similar pathological phenomena in the brain using the rutile form of TiO_2 that we observed with the anatase form.

Although we did not investigate the effect of TiO_2 nanoparticles in the adult animals, it has been reported that TiO_2 nanoparticles do not usually enter the brain of adult

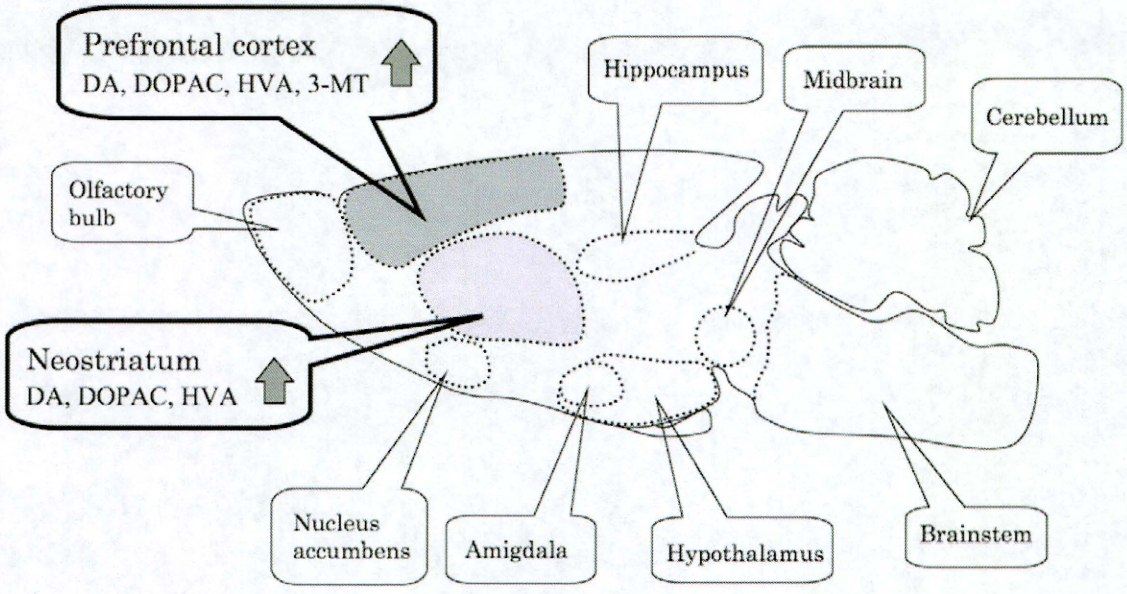

Fig. 3. Summary of changes in the levels of DA and its metabolites in 10 regions of the brain following prenatal exposure to TiO_2 nanoparticles.

animals (Fabian *et al.*, 2008). Since the blood brain barrier is not fully developed in embryos, the developing brain is sensitive to foreign chemicals during the embryonic stage. We have previously reported the penetration of TiO_2 nanoparticles into the brain, and stenosis of peripheral blood vessels of the cerebral cortex and hippocampus in the offspring of female mice exposed to TiO_2 nanoparticles during pregnancy (Takeda *et al.*, 2009). These findings strongly support the hypothesis that prenatal exposure to TiO_2 nanoparticles can affect the development of the central nervous system through the dissemination of nanoparticles into the brain.

TiO_2 nanoparticles were easily aggregated and agglomerated. We also obtained the aggregated TiO_2 nanoparticles around 2 μm in the present study (Fig. 1). However, several investigators have shown that the aggregated TiO_2 nanoparticles around 1.4 μm exerted toxicity (Bermudez *et al.*, 2004; Ferin *et al.*, 1992). Moreover, Sager *et al.* (2008) reported that intratrachial administration of TiO_2 nanoparticles, which agglomerated a diameter of 200-300 nm, cause the pulmonary inflammatory responses. Furthermore, the aggregated/agglomerated TiO_2 nanoparticles have revealed more toxicity than their larger counterparts (Ferin *et al.*, 1992; Sager *et al.*, 2008). These findings propose a hypothesis that TiO_2 nanoparticles may be able to affect the central dopaminergic neuron regardless of aggregation/agglomeration. On the other hand, we observed TiO_2 nanoparticles with a diameter of less than 300 nm in the brain of offspring (Takeda *et al.*, 2009). Recently Wick *et al.* (2010) showed that fluorescent polystyrene particles up to a diameter of 240 nm were taken up and were able to cross the placental barrier without affecting the viability of the explant using the *ex-vivo* human placental perfusion model. Therefore, intact or smaller parts of agglomerate TiO_2 nanoparticles might be able to selectively transfer and affect the DA levels in the brain of offspring.

In conclusion, the present data provide evidence that prenatal exposure to TiO_2 nanoparticles can influence the DA levels of brain in mice (Fig. 3). Further investigation is necessary to fully understand the molecular mechanisms of TiO_2 nanoparticle-mediated alterations of the central nervous system.

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PARTICLE AND FIBRE TOXICOLOGY

RESEARCH Open Access

In utero exposure to a low concentration of diesel exhaust affects spontaneous locomotor activity and monoaminergic system in male mice

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Abstract

Background: Epidemiological studies have suggested that suspended particulate matter (SPM) causes detrimental health effects such as respiratory and cardiovascular diseases, and that diesel exhaust particles from automobiles is a major contributor to SPM. It has been reported that neonatal and adult exposure to diesel exhaust damages the central nervous system (CNS) and induces behavioral alteration. Recently, we have focused on the effects of prenatal exposure to diesel exhaust on the CNS. In this study, we examined the effects of prenatal exposure to low concentration of diesel exhaust on behaviour and the monoaminergic neuron system. Spontaneous locomotor activity (SLA) and monoamine levels in the CNS were assessed.

Methods: Mice were exposed prenatally to a low concentration of diesel exhaust (171 μg DEP/m^3) for 8 hours/day on gestational days 2-16. SLA was assessed for 3 days in 4-week-old mice by analysis of the release of temperature-associated infrared rays. At 5 weeks of age, the mice were sacrificed and the brains were used for analysis by high-performance liquid chromatography (HPLC).

Results and Discussion: Mice exposed to a low concentration of diesel exhaust showed decreased SLA in the first 60 minutes of exposure. Over the entire test period, the mice exposed prenatally to diesel exhaust showed decreased daily SLA compared to that in control mice, and the SLA in each 3 hour period was decreased when the lights were turned on. Neurotransmitter levels, including dopamine and noradrenaline, were increased in the prefrontal cortex (PFC) in the exposure group compared to the control group. The metabolites of dopamine and noradrenaline also increased in the PFC. Neurotransmitter turnover, an index of neuronal activity, of dopamine and noradrenaline was decreased in various regions of the CNS, including the striatum, in the exposure group. The serum corticosterone level was not different between groups. The data suggest that decreased SLA in mice exposed prenatally to diesel exhaust is due to facilitated release of dopamine in the PFC.

Conclusions: These results indicate that exposure of mice *in utero* to a low concentration of diesel exhaust decreases SLA and alters the neurochemical monoamine metabolism of several regions of the brain.

Background

Several epidemiological studies have shown a positive association between the level of ambient particulate matter (PM) and mortality caused by respiratory and cardiovascular diseases [1,2]. Diesel engines produce large amounts of PM, and the health effects of exposure to diesel exhaust have been studied. According to several reports, diesel exhaust and diesel exhaust particles (DEPs), the particulate components of diesel exhaust, can affect the central nervous system (CNS). An epidemiological study showed a group of railroad workers exposed to diesel exhaust had impairment of neurobehaviour [3]. Subsequent studies showed that severe air pollution is associated with brain inflammation, Alzheimer's-like pathology [4-6], disruption of blood-brain barrier [6] and cognitive deficit [7,8]. The exposure of

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human volunteers to ambient levels of DEPs showed abnormal electrical signals of their frontal cortex, the important area for higher brain functions [9]. Animal models have been used to clarify the mechanisms underlying the effects of diesel exhaust exposure. The molecular toxicity of diesel exhaust is suggested to include oxidative stress-mediated inflammation, which is considered to be central to both pulmonary and systemic adverse health effects [10,11]. It was reported that nano-sized DEPs selectively damage cultured dopaminergic neurons by oxidative insult [12] and intracranial microinjection of fractionated diesel exhaust in rat hippocampus and striatum induces tissue damage in these regions [13]. Furthermore, in a recent study, DEPs induced oxidative stress and increased NF-κB at the blood-brain barrier of mice [14]. Another study showed that intranasal administration of nano-sized carbon black particles modulated the increase of expression of inflammatory cytokine mRNA, such as interleukin-1β, and the levels of amino acid neurotransmitters, such as glutamate and glycine, in the mice olfactory bulb induced by lipoteichoic acid [15]. Regarding the effects of prenatal exposure to diesel exhaust (0.3 - 3.0 mg DEP/m^3), numerous caspase-3-positive cells were found in the cerebral cortex and hippocampus of newborn mice [16]. A subsequent study showed that prenatal exposure to diesel exhaust (1.0 mg DEP/m^3) decreased the dopamine turnover, an index of dopamine neuronal activity, in the striatum [17]. However, there are no data to show the effects of prenatal exposure to low concentration of diesel exhaust on the CNS. The present study showed alteration of spontaneous locomotor activity (SLA) and monoamine levels in the CNS of mice following *in utero* exposure to a low concentration of diesel exhaust (0.171 mg DEP/m^3) that corresponds to 1.71-fold of the Japanese environmental quality standard of daily averaged level of suspended particulate matter (SPM).

Methods

Animals

Twenty-six pregnant ICR mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and housed under controlled conditions with 12 hours light/12 hours dark cycle and *ad libitum* access to food and water. They were divided into two groups: diesel exhaust exposure group (n = 12) and control group (n = 14). The mice of the exposure group were exposed to diesel exhaust for 8 hours/day (9:00 - 17:00 h.), for 5 days per week (Monday-Friday) in an inhalation chamber at the Research Institute of Tuberculosis (Japan Anti-Tuberculosis Association, Tokyo, Japan) from gestational days (GD) 2 - 16. After the exposure period, mothers and pups were maintained in a clean room. Pregnant mice delivered their pups on GD 19. The number and the sex ratio of pups in the exposure group and the control group were 114 (male:female = 75:39) and 161 (75:86), respectively. On postnatal day (PND) 4, the number of pups per litter was adjusted randomly to ten. In each group, pups were weaned on PND 21, after which male mice were transported to Tokyo University of Science (Chiba, Japan). Mice were transported carefully to minimize stress factors by Sankyo Labo Service Co., Inc. (Tokyo, Japan) and Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). All experimental animals were handled in accordance with institutional and national guidelines for the care and use of laboratory animals.

Exposure to diesel exhaust

A 2369 cc diesel engine (Isuzu Motors Ltd., Tokyo, Japan) was operated at a speed of 1050 rpm and at 80% load with a commercial oil. The exhaust was introduced into a stainless steel dilution tunnel (450 mm diameter × 6250 mm), where the exhaust was mixed with clean air, and average concentrations of exhaust constituents were maintained at 1.06×10^4 suspended particles/cm^3 (171 $\mu g/m^3$), 1.25 ppm for carbon monoxide (CO), 0.04 ppm for nitrogen dioxide (NO_2), and less than 0.01 ppm for sulfur dioxide (SO_2).

Behavioural analysis

The SLA of each mouse was measured in a transparent acrylic cage (20 cm × 31 cm × 13 cm) with an activity monitor with an infrared ray sensor (NS-AS01; Neuroscience Inc., Tokyo, Japan). The analysis was done when the mice were 4 weeks old (n = 10/group). Movement was measured according to the release of temperature-associated infrared rays. SLA counts were collected at 10 min intervals for 3 days. Data were analyzed automatically with a computerized system (multidigital 32-port counter system; Neuroscience Inc.). The analysis was conducted in "a new environment" and in "a home cage environment", which refers to the first 60 min test period just after moving into a new cage and the subsequent test period each day, respectively. Statistical analysis was done with two-way, repeated-measures analysis of variance (ANOVA), in which the variables were diesel exhaust exposure and time, followed by post hoc Student's t-test. The level of statistical significance was set at $P < 0.05$.

Sampling procedure

Following the behavioural test, brain and trunk blood were obtained from the animals (5 weeks of age). The body weight of the animals was 28.56 - 37.51 g and there was no significant difference in body weight between the exposure group (32.7 ± 2.4 g) and the control group (32.9 ± 2.0 g). The brain was dissected into

six regions, immediately frozen in liquid nitrogen, and stored at -80°C. Serum was separated in a gel barrier capillary blood collection tube (Capiject T-MG; Terumo Medical Corp., Elkton, MD) followed by centrifugation at 2200 *g* at 4°C for 15 min and stored at -80°C until analysis.

Brain dissection

Brain dissection was done according to the modified method of Heffner *et al.* [18] and was based on the atlas described by Paxinos and Franklin [19]. The following four regions were dissected from frozen forebrain and midbrain coronal sections on a silicon plate chilled with dry ice: prefrontal cortex (PFC; containing cingulated cortex and motor cortex areas 1 and 2); striatum (dorsal); hippocampus (caudal) and midbrain (containing ventral tegmental area and substantia nigra). Determination of monoamine levels was done in PFC, striatum, hippocampus, midbrain, cerebellum, and brainstem.

Preparation of homogenates

Frozen brain tissues was homogenized in ice-cold 0.2 M perchloric acid (Nacalai Tesque Inc., Kyoto, Japan) containing 100 μM Na_2-EDTA (Dojinto Laboratories, Kumamoto, Japan) and 1 ng/mL isoproterenol as an internal standard (Sigma-Aldrich Co., St. Louis, MO). The homogenates were kept on ice for 30 min and centrifuged at 20,000 *g* at 0°C for 15 min. The supernatant was mixed with 1 M sodium acetate to adjust the pH to 3.0 (Kanto Chemical Co., Inc., Tokyo, Japan) and were frozen immediately in liquid nitrogen and stored at -80°C. The precipitate was used for the protein assay.

High-performance liquid chromatography (HPLC)

Each group contained samples from 10 mice. A 10 mL sample of the final supernatant was injected with a microsyringe (702SNR; Hamilton Co., Reno, NV) into an HPLC system equipped with an electrochemical detector (HTEC-500MAB; Eicom Co., Kyoto, Japan). The standard solution contained the monoamines dopamine and noradrenaline and their metabolites. The dopamine metabolites were 3-methoxytyramine hydrochloride (3-MT), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). The noradrenaline metabolites were normetanephrine hydrochloride (NM) and 4-hydroxy-3-methoxyphenylglycol hemipiperazinium (MHPG). Standards dopamine, HVA, 3-MT, NM and MHPG were obtained from Sigma-Aldrich. Standards noradrenaline and DOPAC were obtained from Nacalai Tesque and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. Separation of monoamines and their metabolites was done by passage through a C18 reverse-phase column (Eicompak SC-5ODS; 3.0 mm × 150 mm; Eicom), maintained at 25°C and connected to an electrochemical detector (EPC-500, Eicom). The mobile phase was 0.1 M acetic acid/citric acid buffer (pH 3.5) containing Na_2-EDTA (5 mg/L), octanesulfonic acid (190 mg/L; Nacalai Tesque), and methanol (15% (v/v); Kanto Chemical Co., Inc.). The flow rate was maintained at 0.5 mL/min for 35 min. Data were collected and analysed with the PowerChrom 280 System (eDAQ Pty Ltd., New South Wales, Australia). To determine the protein concentration, pellets were dissolved in 100 mM Tris-HCl for protein determination by a high-sensitivity version of the Bradford method with a commercial reagent (ADV-01; Cytoskeleton Inc., Denver, CO), and measurements were done according to the manufacturer's protocol. The absorbance at 595 nm was measured with a 96-well microplate reader (model 550; Bio-Rad Laboratories Inc., Hercules, CA), and protein concentration was calculated from a standard curve generated with bovine γ-globulin (Pre-Diluted Protein Assay Standards: Bovine Gamma Globulin Set; Thermo Fisher Scientific Inc., Rockford, IL). Concentrations of monoamines and their metabolites are expressed as pg mg^{-1} of protein, and the catabolism rate is expressed as the ratio of metabolite to monoamine (e.g. HVA/dopamine). Indices were calculated from individual tissue samples. Statistical analysis was done with the Mann Whitney *U*-test. The level of statistical significance was set at $P < 0.05$.

Measurement of serum corticosterone

The concentration of corticosterone in serum was determined with a Correlate-EIA Corticosterone Enzyme Immunoassay Kit (Assay Designs Inc., Ann Arbor, MI).

Results

Spontaneous locomotor activity (SLA)

SLA was measured continuously for 3 days. During the first 60 min, a decrease in locomotor activity in the mice exposed prenatally to diesel exhaust was found by two-way, repeated-measures ANOVA (Figure 1A). A decrease in daily locomotor activity over the entire testing period was also found (Figure 1B). SLA in each 3 hour period was also altered in the exposure group, and post hoc analysis showed that SLA was decreased at some time points (Figure 1C). During the first day, datapoints 3 and 6 showed significantly decreased values. During the second and third days, datapoint 6 showed decreased values.

The levels of monoamine and their metabolites

The determination of monoamine levels was conducted in six brain regions: PFC, striatum, hippocampus, midbrain, cerebellum and brainstem. In PFC, the levels of dopamine and noradrenaline were increased in the exposure group (Figure 2, 3). The dopamine level in

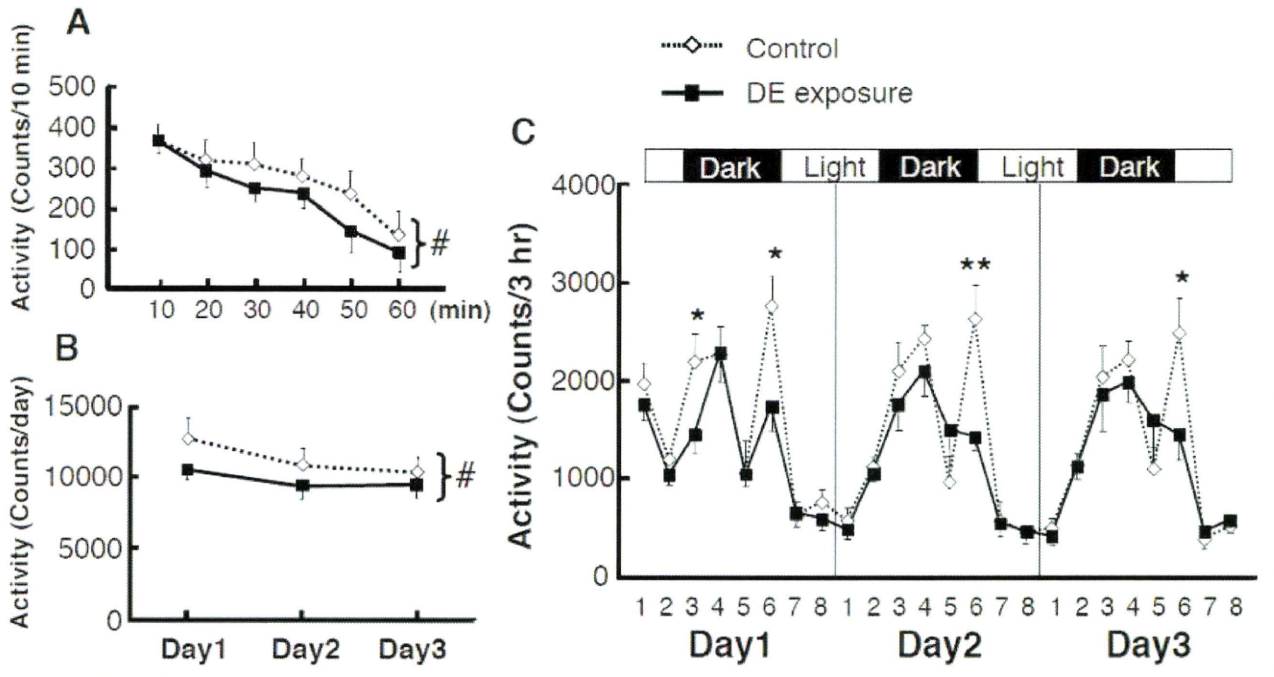

Figure 1 Effect of prenatal exposure to diesel exhaust on spontaneous locomotor activity (SLA) in mice. Each group contained 10 mice. (A) During the initial 60 minutes acclimating to the novel environment, the mice exposed prenatally to diesel exhaust showed lower SLA than the control group. (B) Throughout the testing period, the mice of the exposure group showed lower daily SLA than those of the control group. (C) SLA in each 3 hour period was altered in diesel exhaust-exposed mice. Post hoc analysis revealed that SLA was decreased at some time points. All data are presented as mean ± SEM. The level of statistical significance was set at * $P < 0.05$.

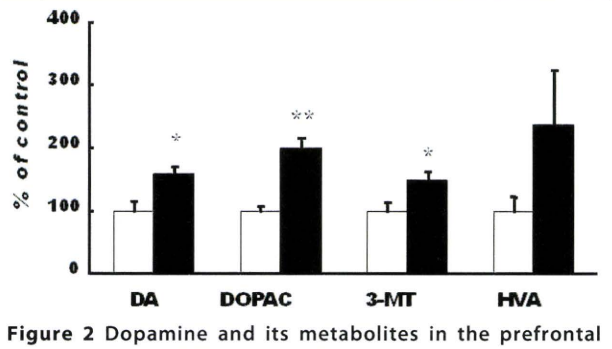

Figure 2 Dopamine and its metabolites in the prefrontal cortex. Dopamine, DOPAC and 3-MT were increased significantly in the exposure group compared to those in the control group. The dopamine turnover (DOPAC/dopamine, 3-MT/dopamine, HVA/dopamine A and [DOPAC + HVA]/dopamine) were not different between the groups. Data are presented as mean ± SEM. The level of statistical significance was set at * $P < 0.05$.

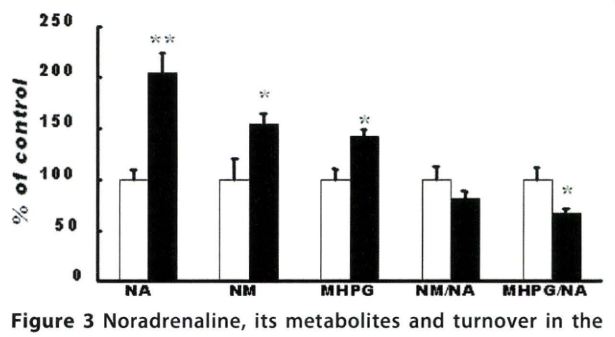

Figure 3 Noradrenaline, its metabolites and turnover in the prefrontal cortex. Noradrenaline, NM and MHPG were increased significantly in the exposure group compared to those in the control group. The noradrenaline turnover (MHPG/noradrenaline) was decreased compared to that in the control group. Data are presented as mean ± SEM. The level of statistical significance was set at * $P < 0.05$.

brainstem was decreased (Table 1) but the dopamine and noradrenaline levels were not altered in the other regions. The levels of metabolites of dopamine were increased in the PFC (Figure 2), and the level of HVA was increased in the brainstem (Table 1). These metabolites were decreased in the hippocampus and in the midbrain (Table 1). The levels of metabolites of noradrenaline were increased in the PFC (Figure 3) but were decreased in the other regions (Table 2). With respect to neurotransmitter turnover, an index of neuronal activity calculated as a ratio of metabolite to transmitter was decreased in some regions of the brain. The turnover of dopamine was decreased in the striatum (Table 3), and that of noradrenaline was decreased in the PFC (Figure 3), striatum, hippocampus, midbrain and cerebellum (Table 4).

Table 1 Amounts of dopamine and its metabolites (pg mg^{-1} protein) in each part of the brain.

Brain region	Group	Content (pg mg^{-1} protein)			
		Dopamine	DOPAC	3-MT	HVA
Striatum	Control	270999 ± 30968	20710 ± 2573	21841 ± 3409	32479 ± 3492
	Exposed	319714 ± 14364	20197 ± 685	22587 ± 1386	31276 ± 1956
Hippocampus	Control	1520 ± 350	N.D.	714 ± 143	23816 ± 8826
	Exposed	951 ± 101	N.D.	449 ± 35	4419 ± 392**
Midbrain	Control	4387 ± 486	2491 ± 191	773 ± 71	3864 ± 225
	Exposed	3446 ± 361	1798 ± 110*	555 ± 40*	2847 ± 238*
Cerebellum	Control	202 ± 29	N.D.	N.D.	3225 ± 1152
	Exposed	197 ± 46	N.D.	N.D.	4367 ± 1343
Brainstem	Control	17959 ± 646	57513 ± 2294	N.D.	2952 ± 252
	Exposed	15775 ± 395*	51445 ± 2244	N.D.	5382 ± 517**

Data are presented as mean ± SEM (n = 10 per group). The statistical significance is shown as *P < 0.05, **P < 0.01; ND, not detectable. Abbreviations: DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 3-MT, 3-methoxytyramine.

Table 2 Amounts of noradrenaline and its metabolites (pg mg^{-1} protein) in each part of the brain.

Brain region	Group	Content (pg mg^{-1} protein)		
		Noradrenaline	NM	MHPG
Striatum	Control	1894 ± 310	N.D.	31671 ± 2828
	Exposed	1983 ± 278	N.D.	23800 ± 1064*
Hippocampus	Control	9872 ± 1149	691 ± 97	44283 ± 5051
	Exposed	10668 ± 625	788 ± 76	30498 ± 1102*
Midbrain	Control	12498 ± 954	410 ± 34	22443 ± 1466
	Exposed	12719 ± 633	357 ± 34	14086 ± 675**
Cerebellum	Control	4203 ± 312	615 ± 60	42898 ± 3439
	Exposed	4174 ± 262	533 ± 45	28305 ± 1730*
Brainstem	Control	457063 ± 21049	25389 ± 1775	189692 ± 11607
	Exposed	430512 ± 18333	20766 ± 1158*	165359 ± 16247

Data are presented as mean ± SEM (n = 10 per group). The statistical significance is shown as *P < 0.05, **P < 0.01; ND, not detectable. Abbreviations: MHPG, 4-hydroxy-3-methoxyphenylglycol; NM, normetanephrine.

Serum corticosterone

The levels of serum corticosterone were not different between the exposure group and the control group (data not shown).

Discussion

This is the first study that has demonstrated the effects of *in utero* exposure to a low concentration of diesel exhaust (0.171 mg DEP/m^3) on locomotor activity and monoamine level in brain tissue. In the present study, we used only male fetuses and pups for analysis because the prevalence of some psychiatric disorders in childhood, such as autism and attention deficit hyperactivity disorder, is higher in men than in women. The results of this study demonstrated that SLA in a new environment and in the home cage environment was decreased and monoaminergic neurochemistry in several regions of the brain was altered in the exposure group. SLA in the home cage environment was particularly decreased when the lights turned on. The alteration of decreased SLA and dopamine turnover in the striatum was similar to a finding in an earlier study that examined the effects of prenatal exposure to a relatively higher concentration of diesel exhaust (1.0 mg DEP/m^3) [17]. The present study showed alteration of the monoamine metabolism in other regions of the CNS, especially the PFC, even by exposure to a lower concentration of diesel exhaust.

It has been reported that diesel exhaust and DEPs contain estrogenic and antiestrogenic compounds and possess endocrine-disrupting activity [20-23]. In the present study, the number of female pups of the exposure group was less than half of that of the control group. It may indicate that embryogenesis of female fetuses was affected by the activity of DEPs. Tsukue *et al.* [24] examined the effects of exposure to diesel exhaust during the perinatal period on sexual differentiation-related gene expression of the brain. Expression levels of *estrogen receptor (ER) α* and *ER β mRNA* were increased in