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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-

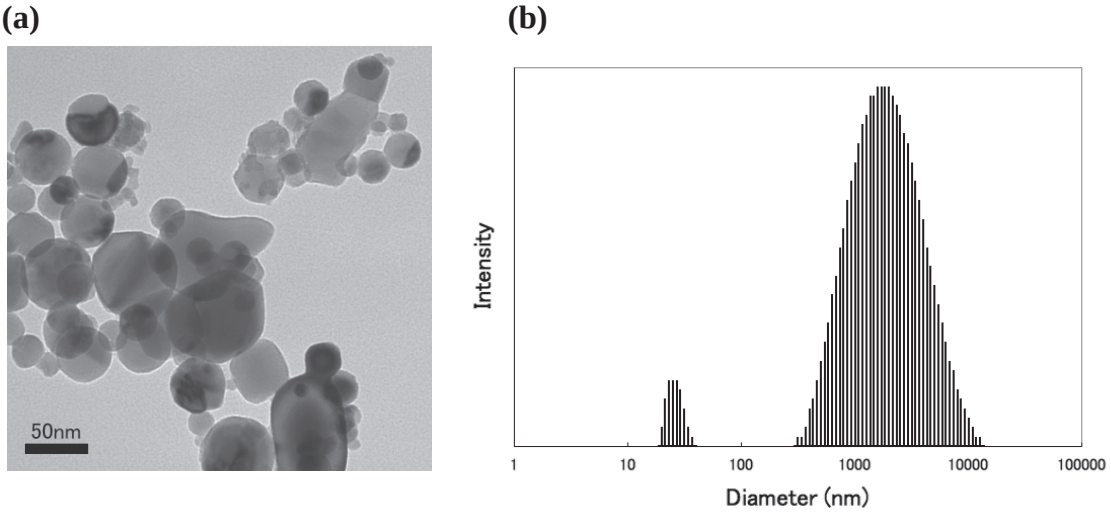

Fig. 1. TEM image of TiO_2 nanoparticles and their size distributions. TiO_2 nanopowder was suspended in saline with 0.05% (v/v) Tween 80 and sonicated for more than 30 min immediately before administration. (a) The agglomeration state was assessed by TEM and (b) the size distribution of the TiO_2 nanoparticles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

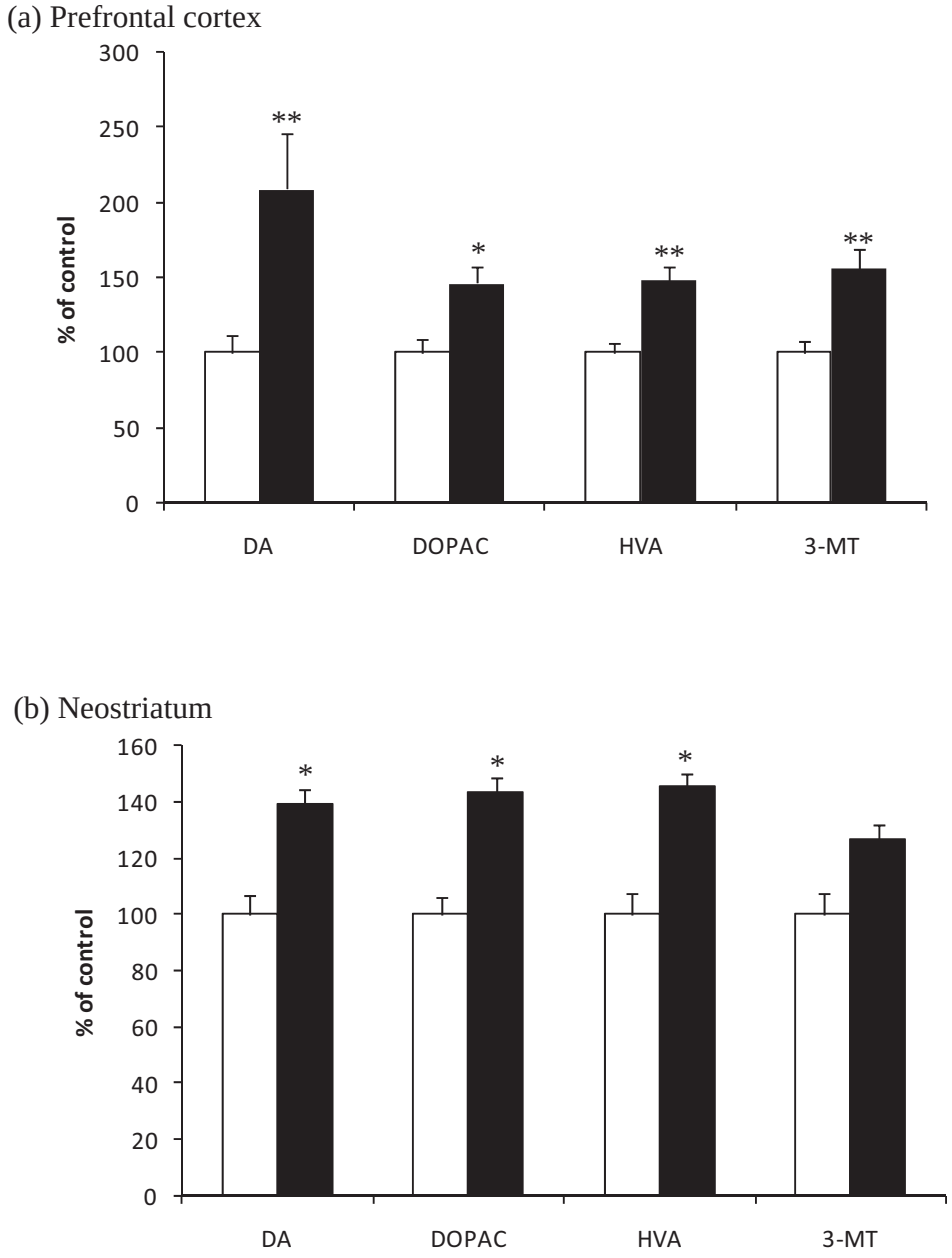

Fig. 2. Changes in the levels of DA and its metabolites (DOPC, HVA, 3-MT) in the (a) prefrontal cortex and (b) neostriatum obtained from 6-week-old male mice exposed, prenatally, to TiO_2 nanoparticles or from control animals. The data are expressed as a percentage of the value in control mice. Each 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

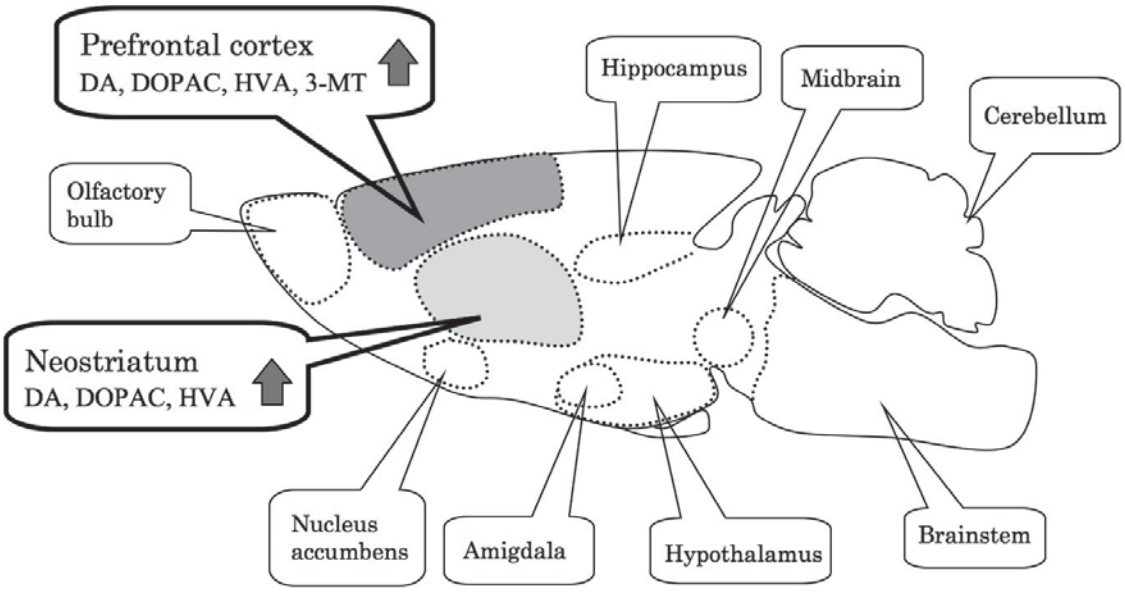

Fig. 3. Summary of changes in the levels of DA and its metabolites in 10 regions of the brain following prenatal exposure to TiO_2 nanoparticles.

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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