

Maternal Exposure to Nano Titanium Dioxide Induces Neurotoxic Effects in Offspring Mice

Fashui Hong^{1,2,3,4*}, Yingjun Zhou^{1,2,3,4}, Jianhui Ji^{1,2,3,4} and Ling Wang⁵

¹Jiangsu Collaborative Innovation Center of Regional Modern Agriculture & Environmental Protection, Huaiyin Normal University, Huaian 223300, China

²Jiangsu Key Laboratory for Food Safety and Nutrition Function Evaluation, Huaiyin Normal University, Huaian 223300, China

³Jiangsu Key Laboratory for Eco-Agricultural Biotechnology around Hongze Lake, Huaiyin Normal University, Huaian 223300, China

⁴School of Life Sciences, Huaiyin Normal University, Huaian 223300, China

⁵Library of Soochow University, Suzhou, China, Suzhou 215123, China

*Corresponding author: Fanwei Meng, Jiangsu Collaborative Innovation Center of Regional Modern Agriculture & Environmental Protection, Huaiyin Normal University, Huaian 223300, China, Tel: + 86-517-83525992; E-mail: hongfsh_cn@sina.com

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Abstract

Although nano-TiO₂ has been extensively used in food industrial application, and in daily products for infants and children, its potential neurotoxicity presents a significant concern. As previous studies have demonstrated the neurological effects of nano-TiO₂ in mice, the main objective of the current investigation was to establish the effects of prenatal exposure of nano-TiO₂ on developing embryos and offspring. ICR female mice were orally administered (1, 2, and 3 mg/kg body weight) nano-TiO₂ from prenatal day 2 to postnatal day 21. Morphological changes, learning and memory, and potential target molecules of neurodevelopmental toxicity in offspring mice of nano-TiO₂-exposed mothers were examined. The results showed that nano-TiO₂ could translocate from prenatally exposed maternal to offspring hippocampi, resulting in hippocampal damage including: a 29.07%-61.4% reduction in spatial recognition; a 22.55%-61.34% reduction in total dendritic length; a 53.57%-71.42% reduction in dendritic branch number; and a 27.72%-75.74% reduction in spine density in offspring CA1 pyramidal cells. Furthermore, expression of several proteins involved in dendritic development was decreased including: a 47.62%-73.81% in microtubule-associated proteins; a 14.58%-54.17% in mitotic kinesin-like protein 1; a 1.14%-31.82% in collapsin response mediator protein 3; a 65.17%-79.05% in neuregulin tyrosine kinase receptor 4; a 1.13%-76.92% in kinesin superfamily protein 17; and a 25.73%-61.4% in post synaptic density protein -95 in offspring hippocampi. Hippocampal neurons may present a major target of neurotoxicity in offspring following maternal exposure to nano-TiO₂ during the prenatal period. Therefore, food application of nano-TiO₂ should be performed with caution.

Keywords: Nanoscale titanium dioxide; Maternal exposure; Offspring mice, Hippocampal development; Development-related protein

Introduction

Owing to its high brightness and refractive index, nano-TiO₂ is commonly used as a pigment in different fields and accounts for almost 70% of the total production volume of all pigments worldwide [1]. Nano-TiO₂ is among the top five nanoparticles used in consumer products, including paints, cosmetics, sunscreens, food products, toothpaste, sterilization, air cleaning and waste water treatment, and articulating prosthetic, hip and knee implants [2-7]. However, nano-TiO₂ has been shown to enter the body and to be deposited in various organs via exposure routes (such as inhalation, injection, dermal and gastrointestinal tract absorption), leading to organ toxicity in mice and rats [8]. For example, Liu et al. reported that following injection of nano-TiO₂ into the abdominal cavity of ICR mice for 14 consecutive days, nano-TiO₂ deposition occurred in various organs in the following order; liver>kidneys>spleen>lung>brain>heart [9]. Although nano-TiO₂ is considered to be physiologically inert to humans, numerous studies to date have reviewed the toxic effects of nano-TiO₂ on the central nervous system (CNS) of mice and rats [8,10-15].

Previous studies suggest that nano-TiO₂ deposited in the mouse hippocampus causes vacuole oxidative damage and cellular degradation in hippocampal neurons, decreased numbers of Nissl bodies in the CA1 region, and increased glial fibrillary acidic protein expression, acetylcholinesterase activity, and glutamate and NO production in the CA4 region, which result in inflammation [16-18]. Furthermore, exposure to nano-TiO₂ promotes calcium deposition in neurocytes and proliferation of ependymal and glial cells, and disrupts the

homeostasis of trace elements, neurotransmitters and enzymes, which lead to oxidative damage, hippocampal apoptosis, and a reduction in spatial recognition memory in mice [19-25].

Importantly, development of humans and animals and in particular, in the developing brain, is significantly influenced by environmental factors [26,27]. Prenatal exposure to nano-TiO₂ is reported to affect the expression of genes related to the development and function of the CNS and to induce elevated caspase 3 levels in the olfactory bulb of mouse brain and in dopamine and its metabolites in offspring mice [28-30]. These detrimental effects have further been shown to decrease hippocampal neuron proliferation and synaptogenesis in addition to learning and memory and long-term synaptic plasticity during development in offspring rats [31-33], and to increase depressive-like behavior during adulthood in forced swimming and sucrose preference tests in offspring rats [34]. However, it remains to be established whether the decrease in offspring spatial recognition memory induced by nano-TiO₂ is associated with disruption of neuronal dendrite development. Here, we examined the hypothesis that nano-TiO₂-mediated reduction of neuronal synaptic plasticity and cognitive function during development in offspring may be related to disruption of dendritic development and influence normal and consequent construction of neural networks.

The mechanism underlying the negative effects of nano-TiO₂ on hippocampal dendritic growth may involve interactions with key proteins related to dendritic development. Accumulating evidence has shown that tubulin, microtubule-associated proteins (MAPs, including MAP2), and apoptosis development-associated proteins, such as mitotic kinesin-like protein 1 (MKLP1), collapsin response mediator protein 3 (CRMP3), neuregulin tyrosine kinase receptor 4 (ErbB4), and kinesin superfamily protein 17 (KIF17), play important roles in dendritic development [35-40]. We were interested in establishing whether these proteins contribute to the neurodevelopmental toxicity effects of nano-TiO₂. ICR female mice were selected as the experimental model and exposed to nano-TiO₂ through oral (gavage) administration from prenatal day 2 to postnatal day (PND) 21. Morphological changes as well as effects on learning and memory were examined in offspring mice following maternal nano-TiO₂ exposure and potential nano-TiO₂ target proteins were identified, with a view of clarifying the specific mechanisms associated with its toxic effects on the developing CNS.

Methods

Chemicals

Nanoparticulate TiO₂ was prepared by controlled hydrolysis of titanium tetrabutoxide. Details of the synthesis and characterization of nano-TiO₂ are described in a previous report by our group [22]. Nanoparticle characteristics were as follows: anatase phase, particle size, 6.5 nm; hydrodynamic diameter, approximately 294 nm; surface area, 174.8 m²/g; and zeta potential, 7.57 mV [22].

Ethics approval

All animal experiments were conducted during the light phase and approved by the Animal Experimental Committee of Soochow University (China) (Ethical approval number 2111270). Procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Animals and treatment

Twenty-four specific pathogen-free (SPF) pregnant mice were purchased from the Animal Center of Soochow University (China) and maintained at the same center in individual cages with free access to water and sterilized food. Pregnant mice were randomly divided into four subgroups (n=6 per group), including a control group treated with 0.5% w/v hydroxypropylmethylcellulose (HPMC; Sigma-Aldrich, St. Louis, MO, USA) and three experimental groups treated with 1, 2, and 3 mg/kg BW nano-TiO₂, respectively, under SPF conditions. For appropriate dose selection, we consulted previous reports [1,24,41]. Animals received 1, 2, and 3 mg/kg body weight (BW) of nano-TiO₂ orally (gavage), respectively, from prenatal day 7 to post-natal day (PND) 21. The same schedule was applied to control pregnant mice treated with 0.5% w/v HPMC. Offspring mice (n=30 per group) delivered spontaneously were reared with their respective dams until the time of initial experimentation at prenatal day 7. Pups were separated from the mother at PND 21 and housed in cages in an isolated animal room under SPF conditions for 21 days. The room environment was set up at 24 ± 2°C under 60% ± 10% relative humidity and a 12 h light/dark cycle.

Behavioral experiment

After PND 21, acquisition of spatial recognition memory was measured in offspring using the Y-maze (n=10 per group). To avoid stress-related interference in the learning procedure, offspring mice were not handled by the experimenter but allowed to voluntarily enter the maze. The Y-maze test for acquisition of spatial recognition memory in offspring mice has been described previously [42,43]. To determine spatial recognition memory, the number of entries and time spent in each arm of the maze by individual offspring mice were recorded, and novelty versus familiarity were analyzed by comparing behaviors in all three arms. The number of arms visited was taken as an indicator of locomotor and exploratory activity [44].

Offspring mice that experienced behavioral experiments were not used for Golgi-Cox staining.

Preparation of hippocampal specimens

After PND 21, all offspring mice were weighed, and sacrificed via cervical dislocation, and brains were quickly removed, weighed, and stored in a nitrogen canister before use, except those for Golgi-Cox staining. After weighing the body and brains, the coefficients of brain mass to BW were calculated as the ratio of brain (wet weight, mg) to BW (g).

Hippocampi were dissected from brains and frozen at -80°C . Every effort was made to minimize animal suffering.

Titanium content analysis

Hippocampal tissues of offspring mice ($n=5$ per group) were thawed, and ~ 15 mg of samples were digested and their titanium contents determined using an inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Elemental X7; Thermo Electron Co., Waltham, MA, USA) [20-22].

Histopathological examination

For pathologic studies, histopathologic examination was performed using standard laboratory procedures. Briefly, hippocampi ($n=5$ in each group) were embedded in paraffin blocks, then sliced ($5\ \mu\text{m}$ thickness) and placed onto glass slides. After hematoxylin-eosin staining, the stained sections were evaluated by a histopathologist, unaware of the treatment, using an optical microscope (Nikon U-III Multi-point Sensor System, Japan).

Golgi-Cox staining procedure

Briefly, Golgi-Cox impregnation and staining and statistical analysis of offspring hippocampi were performed as previously described [45]. Offspring hippocampal sections ($100\ \mu\text{m}$) were cut on a cryostat, mounted onto gelatin slides, cleared with ethanol and xylene, and cover slipped. Morphology was evaluated on coded slides by an observer blinded to the treatment groups using an Olympus BX-51 microscope equipped with a computer-controlled motorized stage and Neurolucida software (MBF Biosciences, Williston, VT, USA).

Western blotting

Total protein was extracted from offspring hippocampal tissues using RIPA buffer (Cell Signaling Technology, Inc., MA, USA) and protein concentrations were determined with a standard bicinchoninic acid protein assay kit (Thermo Fisher Scientific Inc., IL, USA). Protein samples were separated via 8%, 10% or 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. Membranes were initially blocked with phosphate-buffered saline (PBS) containing 5% fat-free dry milk for 2 h and incubated overnight at 4°C with the appropriate primary antibodies. After washing with PBST (a mixture of PBS and 0.05% Tween-20) three times (10 min each), membranes were incubated with secondary HRP-conjugated antibodies for 2 h at room temperature and re-washed three times with PBST (10 min each). Signals were detected using an enhanced chemiluminescence kit (Thermo Fisher Scientific Inc., IL, USA). Western blot was employed to detect expression of post synaptic density protein (PSD)-95, ErBb4, tubulin, KIF-17, MAP2,

MKLP1 and CRMP-3a. Anti-CRMP-3 (C-20)(1:500) and anti-KIF17 (M-20)(1:500) antibodies were obtained from Santa Cruz Biotechnology Inc. (USA), anti-ErBb4 (1:4000), anti-MAP2 (1:2000), anti-Tubulin (1:500) and anti-PSD-95 (1:500) antibodies from EMD Millipore, and anti-MKLP1 (1:2000) antibody from Abcam (Cambridge, UK). Expression of β -actin was detected using an anti- β -actin antibody (diluted 1:2000; Abcam) as the loading control.

Statistical analysis

Data were analyzed using Statistical Analysis Software (SAS 9.1) (Chicago, IL, USA). The significance of differential expression between groups was assessed using one-way ANOVA followed by Tukey's HSD post-hoc test, and results are presented as means \pm standard deviation (SD). Differences were considered statistically significant at $p<0.05$.

Results

Coefficient of brain mass and titanium content

Figure 1 presents the changes in offspring brain coefficients caused by prenatal exposure to nano- TiO_2 . We observed a significant reduction in the brain coefficients in offspring subjected to prenatal nano- TiO_2 exposure ($p>0.05$). Titanium contents in offspring hippocampi are shown in **Figure 2**. With increasing nano- TiO_2 doses, titanium accumulation in offspring hippocampi was markedly increased ($p<0.001$).

Spatial recognition

According to Dellu et al. [43], the two-trial Y-maze task is a specific and sensitive test of spatial recognition memory in rodents, while the primary purpose of the novel arm is to test discriminatory memory. In the latter test, mice have to make a choice between a novel (unfamiliar) and a familiar arm when they are released from the start arm in the Y-maze [43]. The changes in learning and memory of offspring mice induced by nano- TiO_2 are depicted in **Figure 3**. The results showed that offspring mice exposed to nano- TiO_2 had a higher percentage duration in the novel arm relative to that in the start and other arms than those in the control group. Notably, duration in the novel arm of offspring from 1, 2 or 3 mg/kg nano- TiO_2 -treated pregnant mice was lower compared to that of offspring from control pregnant mice ($p<0.05$), indicating that prenatal exposure to nano- TiO_2 decreased spatial recognition memory in offspring. Locomotor activity is a function of the level of excitability of the CNS [44]. Changes in offspring locomotor activity (maze arm visits) affected by nano- TiO_2 are presented in **Figure 4**. The collective findings indicate that prenatal exposure to nano- TiO_2 decreased the number of arm entries in offspring mice ($p<0.05$).

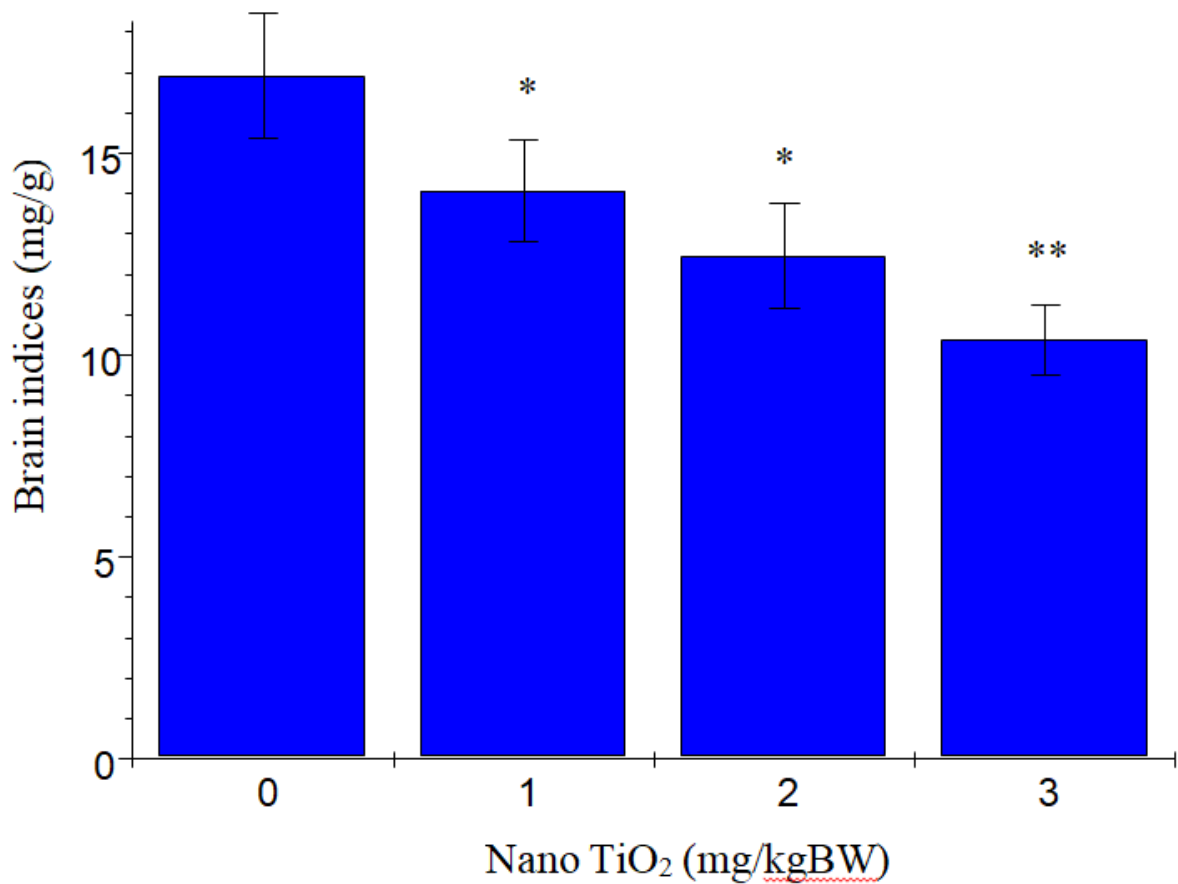


Figure 1: Alterations in the coefficients of brain to body weight in offspring mice following prenatal exposure to nano-TiO₂. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Values represent means \pm SD (n=30).

Histopathological observations

Figure 5 shows the histopathological changes of the hippocampus in offspring mice induced by prenatal exposure to nano-TiO₂ at PND 21. In the control group, the wide spaces among the neurons, glial edema, normal pyramidal cell layer structure and axon structure were clearly observed. However, the hippocampal pyramidal cells in nano-TiO₂ groups decreased and became thinner; the pyramidal cell arrangement was disordered; and pyramidal cells were denatured, necrotic and detached. Empty neuropils were also observed, suggesting a decrease in nerve fiber tracts (**Figure 5**).

Hippocampal neuron development

Morphological analysis was performed on a total of 500 hippocampal CA1 pyramidal neurons from ten offspring mice in each group. The Golgi-Cox impregnation procedure clearly filled the dendrite shafts and spines of pyramidal neurons from the hippocampal CA1 subfield in control offspring mice (**Figure 6**).

However, in the prenatal nano-TiO₂-treated offspring group, we observed a lower extent of Golgi-Cox impregnation of dendrite shafts and pyramidal neuronal spines (**Figure 6**). Data on total dendritic length and spine density were obtained from both sides of the hippocampus in offspring mice (**Figure 7**). CA1 pyramidal neurons from the prenatal nano-TiO₂-treated groups showed significant atrophy in dendritic length compared with those of the control offspring group, with the 1, 2, and 3 mg/kg treatment groups showing a 22.55%, 39.24% and 61.34% reduction in total dendritic length, respectively (**Figure 7**, $p < 0.05$). Compared with control offspring, the dendritic branches and spine density on dendritic trees of CA1 pyramidal cells were significantly decreased in the prenatal nano-TiO₂-treated groups, with a 53.57%, 57.14% and 71.42% reduction in dendritic branch number and a 27.72%, 57.87%, and 75.74% reduction in spine density, respectively (**Figure 7**, $p < 0.05$). Clearly, prenatal exposure to nano-TiO₂ severely inhibits hippocampal neuron development in offspring mice.

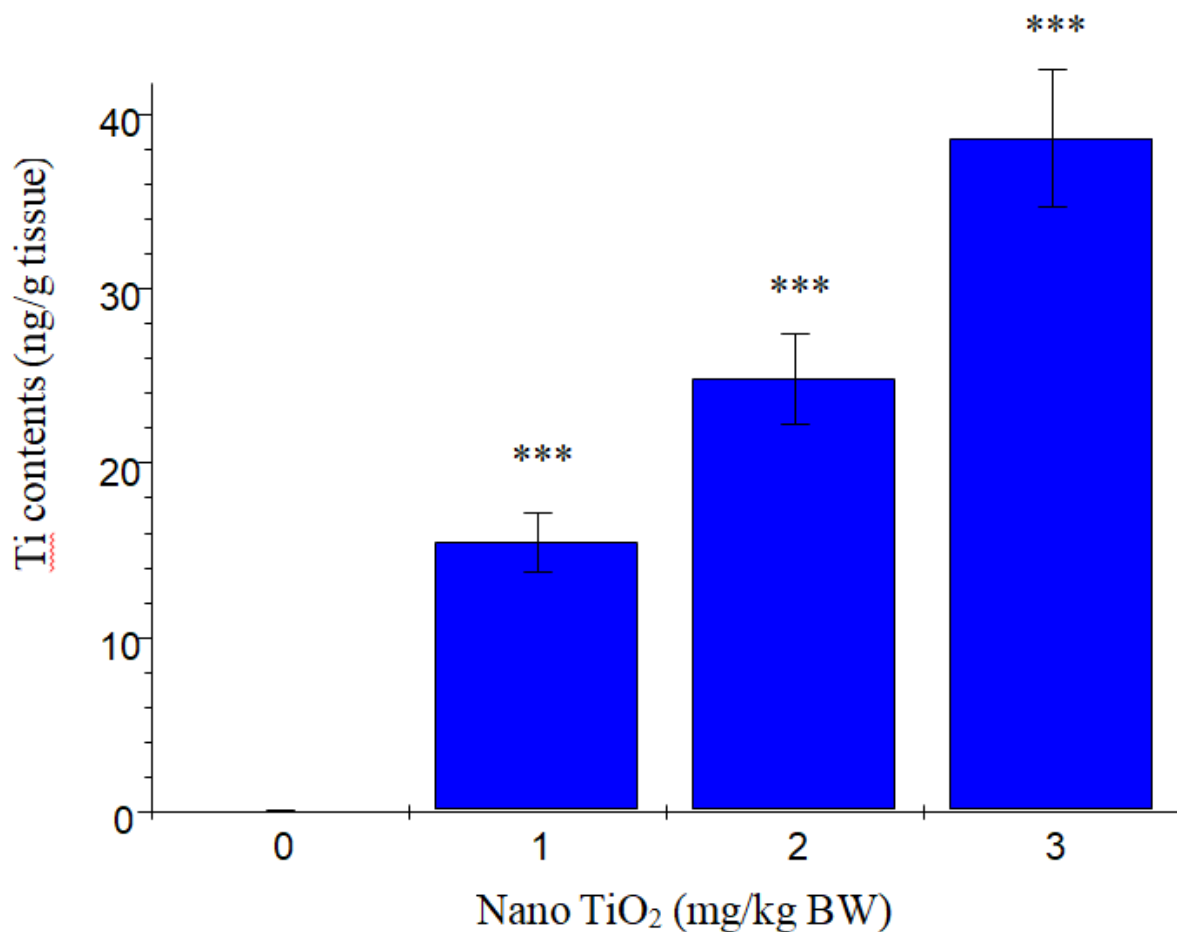


Figure 2: Changes in titanium contents in offspring mouse brain due to prenatal exposure to nano-TiO₂. *** p < 0.001. Values represent means \pm SD (n=5).

Expression of dendritic development-related proteins

To determine the precise molecular mechanisms underlying suppression of hippocampal neuronal development in offspring mice, expression patterns of neuron outgrowth-related proteins were examined using western blot (**Figure 8**). Prenatal exposure to nano-TiO₂ markedly suppressed the expression of MAP2, Erbb4, CRMP-3a, KIF-17, MKLP-1, and PSD-95 (**Figure 8**, p<0.05) but had no significant effect on tubulin expression in the hippocampus of offspring mice (**Figure 8**, p>0.05).

Discussion

Studies to date demonstrate that prenatal exposure to nano-TiO₂ decreases cognitive function in offspring rats but have not focused on dendritic development and the underlying molecular mechanisms associated with this process [31-34]. Recently, we reported that exposure to nano-TiO₂ in vitro resulted in significant suppression in neurite growth in primary cultured

hippocampal neurons [46,47]. In the current study, we investigated the effects of prenatal exposure to nano-TiO₂ on the development of hippocampal dendritic morphology and the associated molecular mechanisms related to this exposure in offspring mice. Our data suggest that in mice prenatally exposed to nano-TiO₂, nanoparticles translocate from the maternal to the offspring brain (**Figures 2 and 5**), demonstrating that nano-TiO₂ can cross the fetal blood barrier. Due to the translocation of nano-TiO₂, prenatal exposure leads to significant reductions in brain coefficients (**Figure 1**) and spatial recognition (**Figures 3 and 4**); increases hippocampal damage (**Figure 5**); alters pyramidal neuron dendritic spines and shafts; and reduces dendritic branching and spine density in CA1 pyramidal cells in the offspring hippocampus (**Figures 6 and 7**). Based on these results, we propose that decreased learning and memory are closely associated with suppression of dendritic development, which may have been triggered by changes in dendritic development-related protein expression in offspring mice as a result of prenatal exposure to nano-TiO₂.

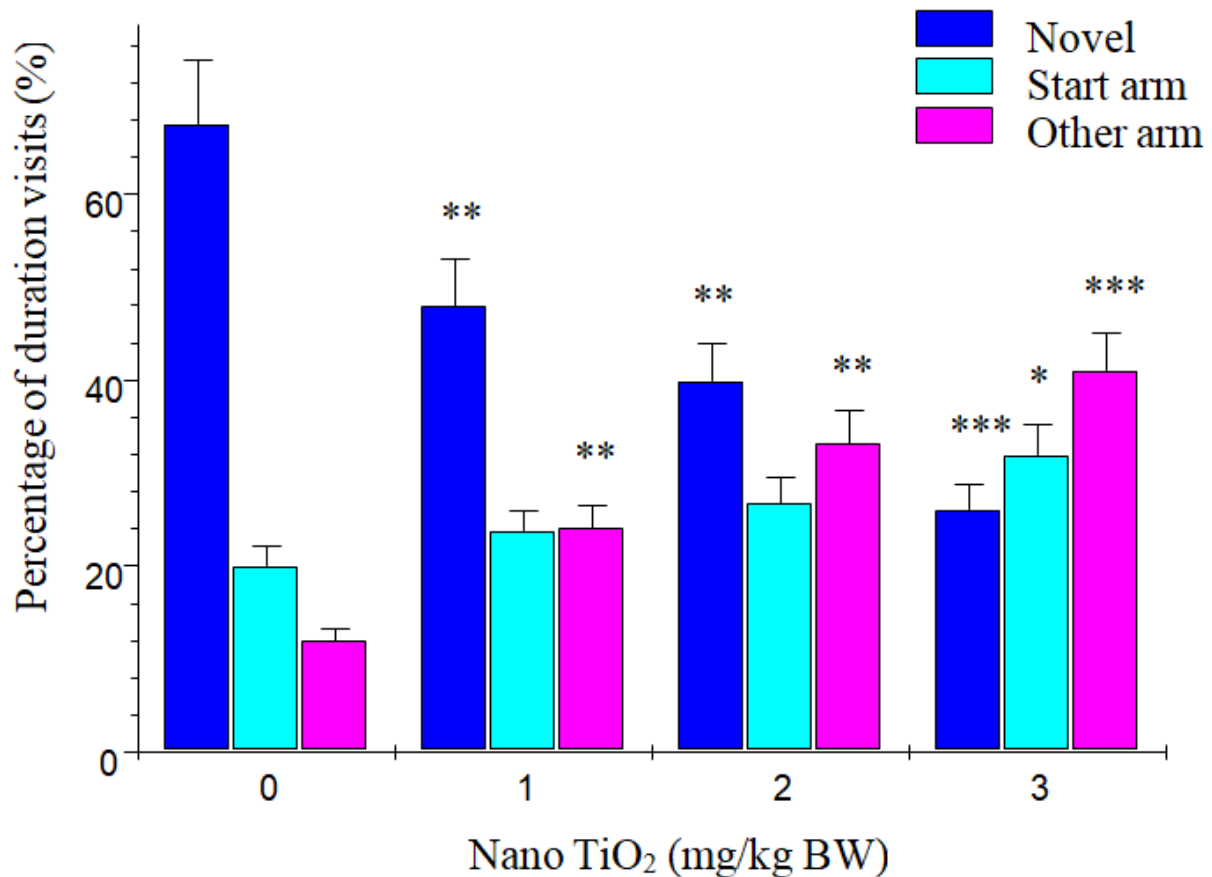


Figure 3: Effect of prenatal exposure to nano-TiO₂ on spatial recognition memory in offspring mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Values represent means \pm SD ($n=10$).

Neurons are polarized cells, with axon and dendrites at opposing sides, which determines polarity during development. In hippocampal neurons, the branch of apical dendrites plays a key role in the process of accepting incoming information, axons determine the scope and breadth of information spread, and basal dendrites play a crucial role in the feedback loop formation. Therefore, the dendritic shape is critical for neurons receiving input, with tree branching being critical for the formation of neural circuits. The critical period of brain development is during pregnancy and the early postnatal stage. The period of exposure to nano-TiO₂ may thus influence neuronal development and the normal neural network. The mechanism of nano-TiO₂-mediated suppression may be related to its regulatory effects on proteins that function in brain development. Tubulin and MAPs are known to be important foundation materials for neurite growth. Among these, MAP2, a heat-stable protein of the structural MAP family, is mainly expressed in neuronal somata and dendrites of the CNS and are believed to interact with and to promote microtubule stability. MAP2 may also be involved in neuronal development and structural stability, the formation apophysis, and in the regulation of synaptic plasticity [35]. In addition to MAPs,

numerous key molecules involving neuronal processes and development and apophysis development have been identified, including MKLP1, CRMP3, ErbB4, and KIF17 [36-40]. Inhibition of MKLP1 expression not only suppresses the development of neuronal dendrites but also results in loss of characteristics of complete morphology and of molecular composition of dendrites, ultimately leading to loss of dendritic function [36]. CRMP3 is an important brain-specific protein upstream of serine/threonine protein kinase activation that acts as an intracellular signaling molecule. IT influences cone neurite morphology by directly regulating cytoskeletal integrity and participates in the establishment of synaptic structure and neural networks [37]. An earlier study by Mechawar et al. demonstrated that ErbB4 is expressed by neuronal somatodendrites in the cerebral cortex and hippocampus from birth to adulthood, supporting a role for neuregulins in dendritic growth and plasticity [48]. Selective ablation of ErbB4 from excitatory neurons leads to a reduction in the proportion of mature spines and overall dendritic spine density in the prefrontal cortex of weanling (PND 21) mice that persists at 2 months of age [49].

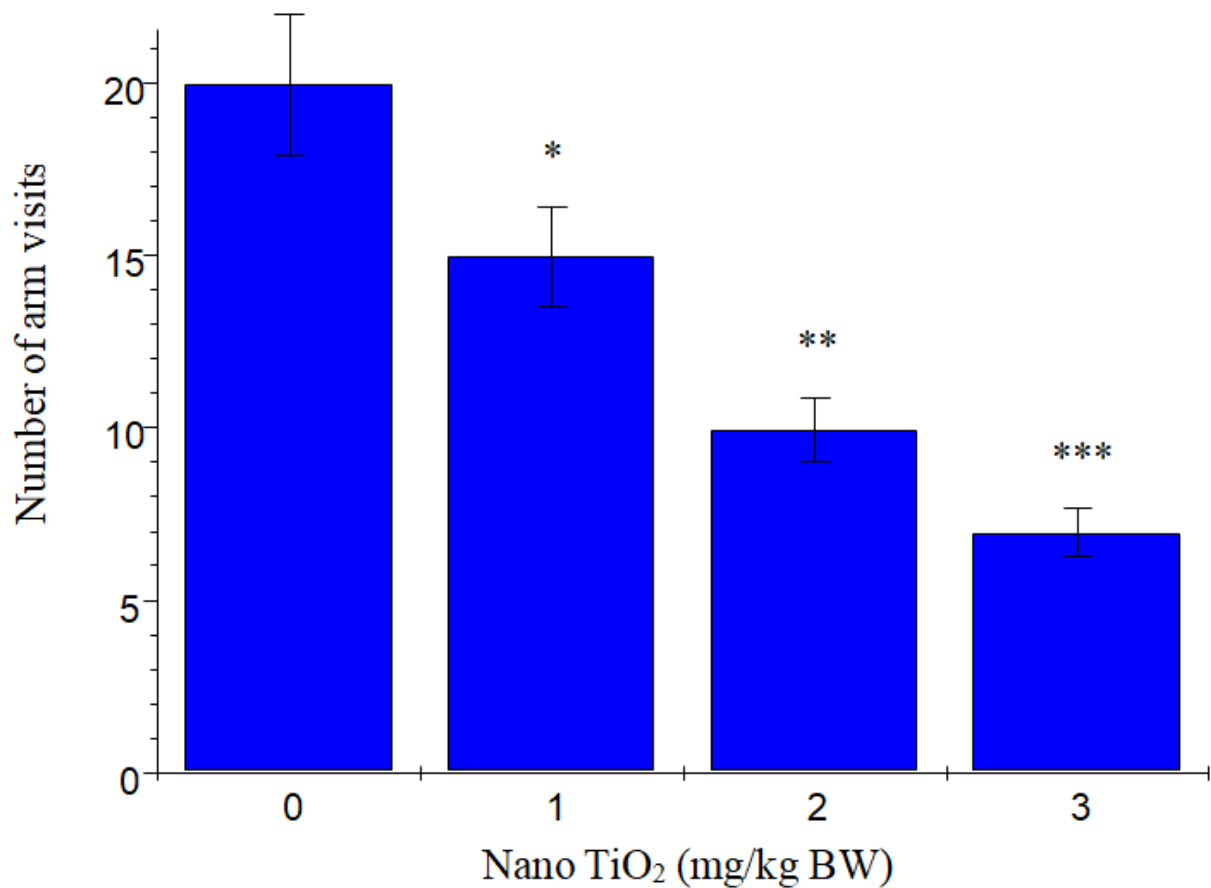


Figure 4: Effect of prenatal exposure to nano-TiO₂ on locomotor activity in offspring mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Values represent means \pm SD (n=10).

These results suggest that ErbB4 signaling in excitatory pyramidal cells is critical for the proper formation and maintenance of dendritic spines. In highly polarized cells such as neurons, sorting and delivery processes of organelles depend on the KIF protein family [50,51]. KIF17, a member of the kinesin superfamily of proteins, plays a fundamental role in spatial learning and memory [52, 53]. Zhao et al. demonstrated that prenatal stress leads to long-lasting deterioration of the expression and function of hippocampal KIF17 in offspring, which may be related to deficits in spatial cognition [54]. Importantly, in the context of dendritic transport in neurons, KIF17 has been implicated in trafficking of glutamate receptors to the synapse [40,55,56]. In the present study, prenatal exposure to nano-TiO₂ induced a significant decrease in the

levels of MAP2, MKLP1, CRMP3, ErbB4, and KIF17 but had no marked effect on tubulin protein expression in the hippocampus of offspring mice (**Figure 8**). Our findings suggest that suppression of dendritic development in offspring mice prenatally exposed to nano-TiO₂ is closely associated with a reduction of MAP2, MKLP1, CRMP3, ErbB4, and KIF17, which in turn leads to decreased learning and memory. Additionally, prenatal exposure to nano-TiO₂ significantly inhibited PSD-9 expression (**Figure 8**). PSD-95 is expressed in central synapses and contributes to synapse and dendritic development [57]. Accordingly, developmental disorders of hippocampal neurons in offspring mice may also be associated with downregulation of PSD-95 induced by prenatal exposure to nano-TiO₂.

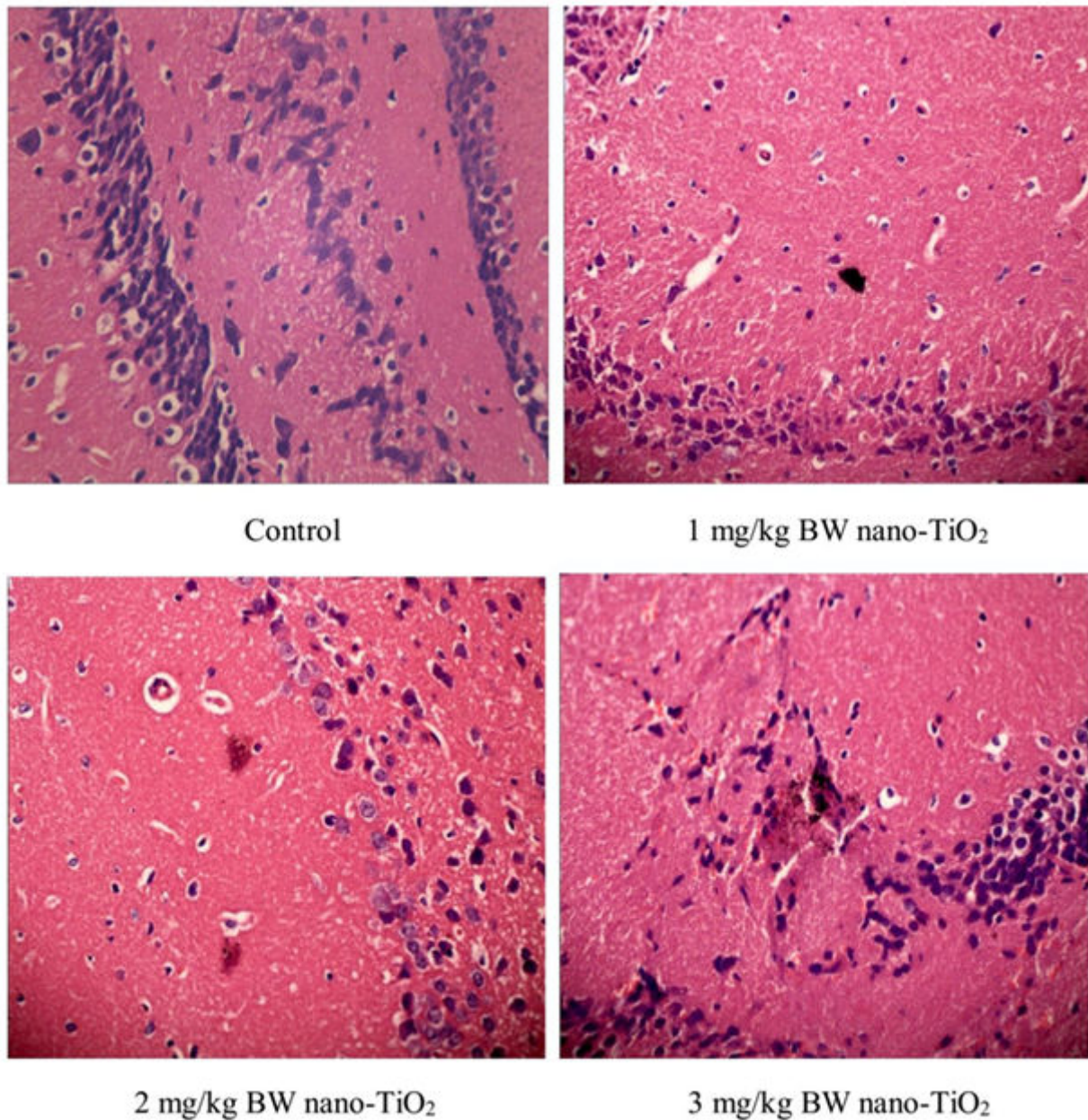


Figure 5: Histopathology of the hippocampal CA1 area in offspring mice following prenatal exposure to nano-TiO₂ after postnatal day 21 (scale 25 μ m). Control group indicated the wide spaces around the neurons, the light glial edema, the normal structure of pyramidal cell layer, and the clear and recognizable axon. 1 mg/kg BW nano-TiO₂ group indicated nano-TiO₂ deposition in hippocampus, light glial edema, disorder of pyramidal cell arrangement, sparse and degeneration of glial cells. 2 mg/kg BW nano-TiO₂ group indicated nano-TiO₂ deposition in hippocampus, glial edema, sparse and degeneration of glial cells, reduced pyramidal cell volume, an irregular pyramidal nucleus, and a large number of denatured pyramidal cells. 3 mg/kg BW nano-TiO₂ group indicated nano-TiO₂ deposition in hippocampus, empty neuropil, pyramidal cell necrosis, shedding and scattered.

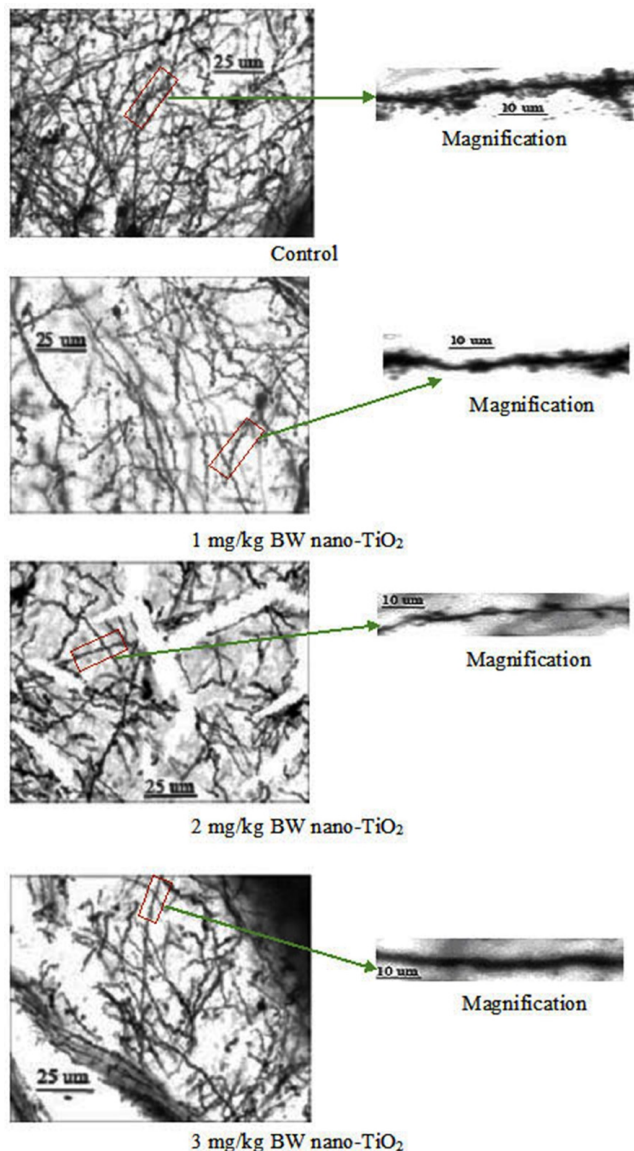


Figure 6: Photomicrograph showing representative Golgi-Cox impregnated pyramidal neurons in the hippocampal CA1 area of offspring mice prenatally exposed to nano-TiO₂ at PND 21.

As discussed, it may be inevitable that people are exposed to nano-TiO₂, and measures should be considered to treat possible nervous system injuries associated with this exposure. Nutraceuticals or diet elements have been demonstrated to improve human health [58], and it may be possible to decrease nano-TiO₂ induced neurotoxicity by consuming nutraceuticals or diet elements.

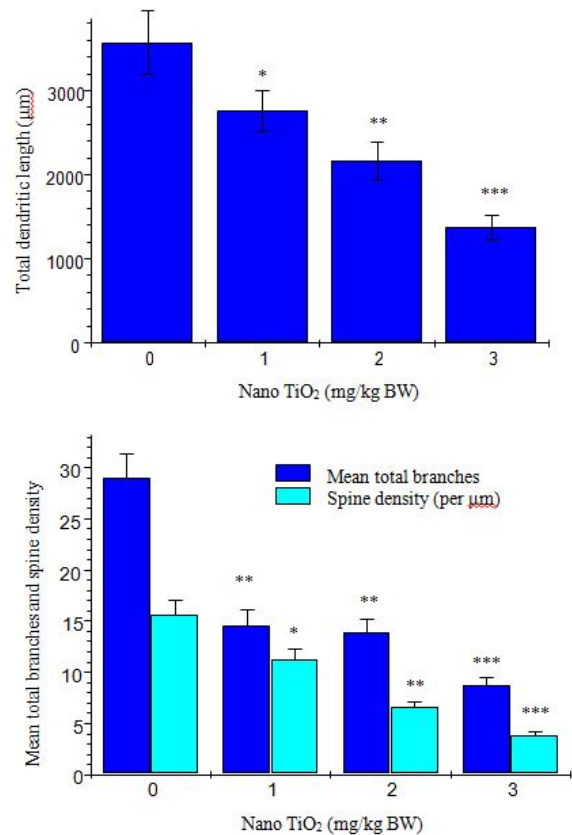


Figure 7: Morphometric analysis of dendrites in CA1 pyramidal neurons of offspring mice prenatally exposed to nano-TiO₂ at PND 21. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Values represent means \pm SD ($n=10$).

Conclusions

Prenatal exposure to nano-TiO₂ results in deposition in the offspring brain, resulting in decreased learning and memory in offspring mice, which appears to be related to hippocampal damage and suppression of hippocampal dendritic development. Furthermore, expression of dendritic developmentally related proteins, including MAP2, MKLP1, CRMP3, ErbB4, KIF17 and PSD-95, is inhibited in mice prenatally exposed to nano-TiO₂, suggesting that disruption of hippocampal neuronal development in offspring mice is triggered via altered regulation of proteins involved in dendritic development. A schematic depicting the proposed mechanism of TiO₂ NP-mediated suppression of neuron dendritic development in offspring mice is presented in **Figure 9**. In view of these findings, we recommend that application of nano-TiO₂ in food, cosmetics, sunscreens, toothpastes, and daily products for infants and children be performed with caution.

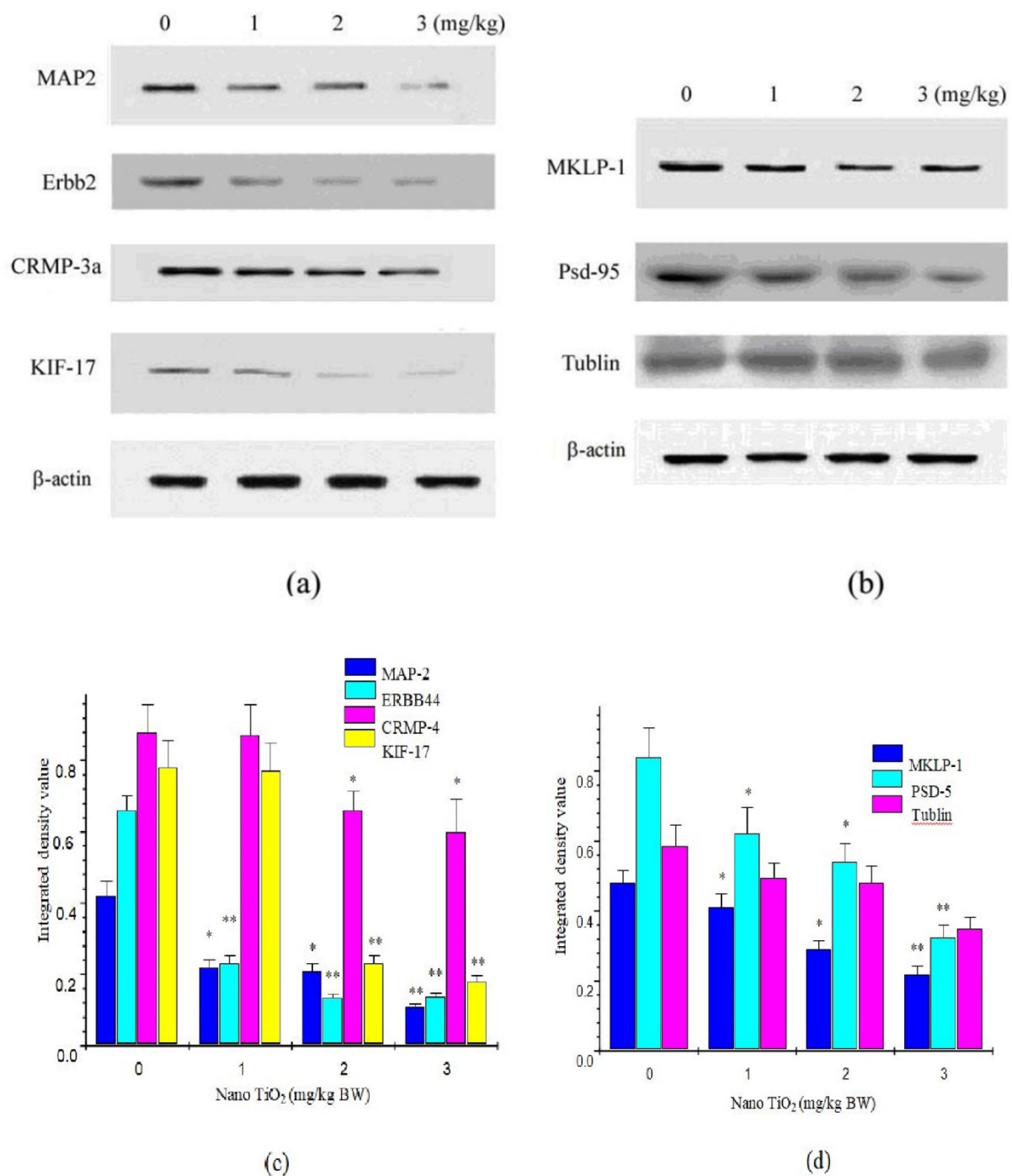


Figure 8: Effects of prenatal exposure to nano-TiO₂ on expression of neuron outgrowth-related proteins in offspring hippocampus. (a), (b) Representative western blots. (c), (d) Expression patterns of neuron outgrowth-related proteins. *p < 0.05, **p < 0.01, ***p < 0.001. Values represent means ± SD (n=5).

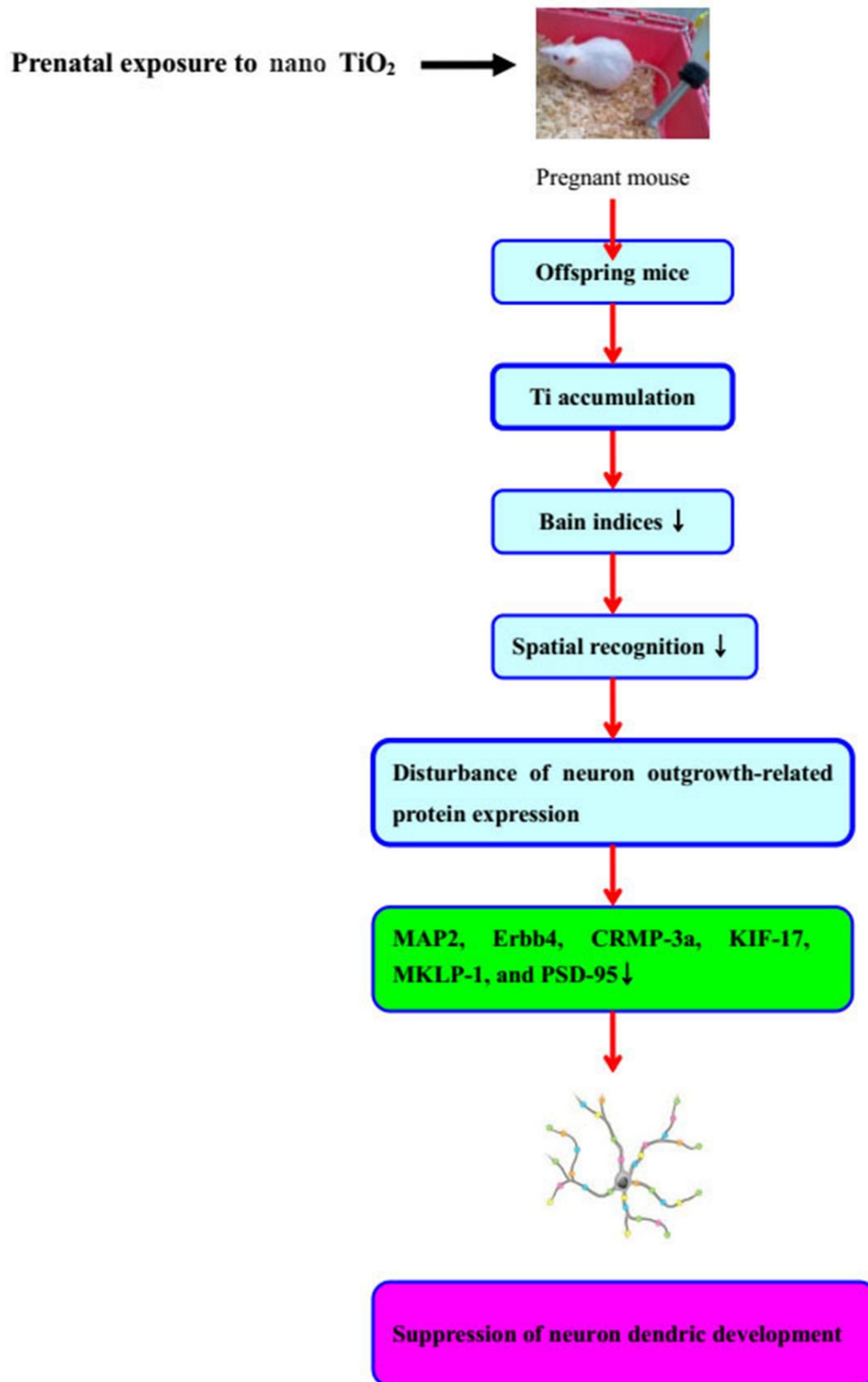


Figure 9: Schematic representation of the potential mechanism underlying nano TiO_2 suppression of neuron dendrite development in offspring mice.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

FH contributed to the design of the whole study and the proof reading of the paper. FH, LW and YJ contributed to the experiments of mice, morphology examination, assays of behavioral experiment, protein expression, and TiO₂ NPs contents. All authors read and approved the final manuscript.

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