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Brain Region and Sex-specific Changes in Mitochondrial Biogenesis Induced by Acute Trimethyltin Exposure

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Objective: In this study, we investigated sex- and region-specific effects of acute trimethyltin (TMT) exposure on mitochondrial biogenesis.
Methods: We treated TMT to primary neuronal cultures and 4-week-old male and female mice. We measured the mitochondrial DNA copy numbers using the quantitative polymerase chain reaction method. We also measured mitochondrial biogenesis related genes (sirtuin-1, estrogen-related receptor alpha, cytochrome C oxidase subunit IV) by western blotting.
Results: The mitochondrial DNA copy number increased in the primary hippocampal neuron; however, it decreased in the primary cortical neuron. The mitochondrial copy number increased in the hippocampus and decreased in the cortex in the TMT treated female mice, though the mitochondrial copy number increased in both cortex and hippocampus in the TMT treated male mice. TMT treatment increased sirtuin-1 expression in the male hippocampus but did not in the female brain. In the female brain, estrogen-related receptor alpha expression decreased in the cortex though there is no significant change in the male brain. The protein level of mitochondrial protein, cytochrome C oxidase subunit IV, increased in both cortex and hippocampus after TMT injection in male mice brain, but not in female mice brain.
Conclusion: Our data suggest that acute TMT exposure induces distinct sex-specific metabolic characteristics in the brain before significant sexual maturation.

KEY WORDS: Trimethyltin; Mitochondria; Estrogens; Sex characteristics.

INTRODUCTION

Trimethyltin (TMT) is an organotin compound that induces a distinct pattern of selective neuronal toxicity in the central nervous system [1]. The neuropathological, neurochemical, and neurobehavioral alterations exerted *in vivo* have been analyzed in rodents, and human cases of accidental poisoning. In particular, the role of oxidative stress in TMT-mediated toxicity has been previously demonstrated by different investigators, indicating the direct involvement of mitochondrial function in response to the neurotoxicant [2]. TMT is likely to act as an environmental toxin for all ages as well as adults [3]. Therefore, research on TMT toxicity in adolescence is needed.

Mitochondria generate adenosine triphosphate (ATP) and are crucial regulators of various cellular processes. Disturbances in mitochondrial-mediated functions, including mitochondria-mediated oxidative stress and changes in intracellular calcium homeostasis, are related to human pathophysiology [4-6]. By utilizing complex mechanisms, cells finely tune mitochondrial numbers. Moreover, several different signaling cascades and transcriptional complexes are involved in mitochondrial biogenesis and functions. The appropriate control of mitochondrial biogenesis against neurotoxic stimuli is crucial for a neuronal survival. In many diseases, mechanisms related to mitochondrial biogenesis are reportedly perturbed [7-9].

There are sexual differences in the mitochondrial respiratory function in physiologic conditions [10-12]. According

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to several studies, the harmful effects of neurotoxic environment changes depend on sex [13,14]. In particular, mitochondria's sexual difference is potentially vital in the effects of early life stress on brain function [15,16]. However, there is no study on whether there are sexual differences in the effects of TMT on adolescent brain mitochondria.

In the present study, following TMT administration, we investigated the regulation of the mitochondrial biogenesis in neuronal cell cultures, as well as in the 4-week-old brains of female and male mice before the maturation of sexual hormones.

METHODS

Cell Culture

We purchased mouse hippocampal (M-Hi-401) and cortical (M-Cx-400) primary neuronal cells from Lonza (Basel, Switzerland). Each primary cell culture was maintained in Neural Progenitor Maintenance Medium (Lonza), supplemented with 2% Neural Survival Factor-1, 0.2% gentamicin/amphotericin-B, and 25 ng/ml Brain-Derived Neurotrophic Factor and Primary Neuron Growth Medium Kit (Lonza), respectively. All cells were incubated at 37°C in the presence of 5% CO_2.

Animal Model

We purchased three-week-old C57BL/6 mice from Samtako Bio Korea (Osan, Korea). We adapted mice in rooms with adequate humidity (50 ± 10%) and temperature (22 ± 2°C) and supplied water and food freely. After one week, we intraperitoneally administered either 2.3 mg/kg TMT in a vehicle (normal saline) or a vehicle. We administered 20 ml/kg solutions to all groups. After 24 hours, we sacrificed mice with isoflurane (Hana Pharm, Seoul, Korea) anesthesia, excised brains, and stored samples at −80°C. The number of mice used in the study was 31, and we used cortical tissue and hippocampal tissue in each mouse. The total number of tissues used is 62. We conducted all procedures following the guidelines of the Eulji University Institutional Animals Care and Usage Committee (IACUC, document number: EUIACUC-17-12) and the ARRIVE guide for the care and use of laboratory animals.

Mitochondrial DNA (mtDNA) Copy Number Assay

Primary mouse neuronal cells were plated onto coated 24-well plates, stabilized for 10 days, and then treated with 5 μM TMT for 72 hours. Total cellular DNA, as well as tissue DNA from the TMT mouse model, were extracted using DNeasy blood and tissue kit (Qiagen, Germantown, MD, USA). Quantitative polymerase chain reaction (qPCR) was performed using the iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) in a CFX96 Real-Time System (Bio-Rad). The relative mtDNA copy numbers were determined using the $2^{-\Delta\Delta Ct}$ method. Beta-globin (HBB) and β-actin genes were used as controls for the primary cell and mouse samples, respectively. HBB primer sequences were as follows: forward, 5′-GCCTGATTCCGTAGAGCCAC-3′; reverse, 5′-CACCCA ACTTCTTCTTGTGA-3′. β-actin primer sequences were; forward, 5′-GATGCCACAGGATTCCATACCTA-3′; reverse, 5′-AGCCTAGTCCTTTCTCCATCTAAAG-3′. mtDNA primer sequences were; forward, 5′-GCCCATGACCAACAT AACTG-3′; reverse, 5′-CCTTGACGGCTATGTTGATG-3′.

Western Blotting

We isolated the total protein of mice's brain cortex and hippocampus using the RIPA buffer (ATTO, Tokyo, Japan). We measured concentrations using the bicinchoninic acid assay. We loaded 20 μg of cytosolic protein per well, separated on 10% SDS-PAGE gels, and transferred on nitrocellulose membranes (Pall, Port Washington, NY, USA). We blocked membranes with 5% non-fat milk in Tris-buffered saline, 0.1% Tween 20 (TBST) buffer for one hour at 4°C and incubated with primary antibodies at 4°C overnight. The primary antibodies were as follows: cytochrome C oxidase subunit IV (COX4), sirtuin 1 (SIRT1), estrogen-related receptor alpha (ERRα) (Cell Signaling Technology, Danvers, MA, USA), and CYP1B1 (Abcam, Cambridge, MA, USA). Then, we rinsed membranes with TBST and incubated them with horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for one hour. After washing with TBST buffer, we incubated the membranes with West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA). We detected protein expression levels by exposure to an X-ray film (Agfa, Mortsel, Belgium) and analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

We conducted all statistical analyses using IBM SPSS Statistics for Windows version 20.0 (IBM Co., Armonk, NY, USA). We expressed quantitative results as mean ± standard deviation, and groups were compared using Dunnett's test and unpaired *t* test.

RESULTS

Acute TMT Exposure Affects mtDNA Copy Numbers in Mouse Primary Neuronal Cells and Mice Brain Tissue

First, we treated 5 μM of TMT in primary hippocampal and primary cortical cultures and measured the mtDNA copy number using qPCR. TMT treatment decreased mtDNA copy numbers ($p < 0.05$) (Fig. 1A). In contrast, in hippocampal neurons, mtDNA copy numbers increased ($p < 0.05$) (Fig. 1B). In primary neuronal cultures, TMT affected the regulation of mitochondrial biogenesis, revealing regional specificity.

Additionally, we administered TMT intraperitoneally (2.3 mg/kg) to 4-week-old female and male mice and measured the mtDNA copy number in cortical and hippocampal tissues. Interestingly, following TMT administration, the mtDNA copy number increased in the male cortex ($p < 0.05$), but decreased in the female cortex ($p < 0.01$) (Fig. 1C). In the hippocampus, TMT increased the mtDNA copy number in both male and female mice (male: $p < 0.05$, female: $p < 0.001$) (Fig. 1D). These findings indicated that TMT altered the regulation of mitochondrial biogenesis, presenting sex and regional differences.

Sex Differences in Gene Expression for Mitochondrial Biogenesis in Response to TMT with Brain Region Specificity

To specifically assess changes in mitochondrial biogenesis following TMT treatment, we measured the levels of mitochondrial biogenesis related genes, SIRT1 and ERRα, using western blotting. In male mice's cortex, protein levels of ERRα and SIRT1 did not alter significantly following TMT treatment (Fig. 2A). In the male hippocampus, TMT treatment significantly increased the SIRT1 protein level ($p < 0.01$); however, the protein level of ERRα did not change significantly (Fig. 2B). In the female mice cortex, SIRT1 levels did not change, but ERRα levels decreased after TMT treatment ($p < 0.05$) (Fig. 3A, Supplementary Fig. 1; available online). In the female hippocampus, the ERRα level did not alter following TMT administration (Fig. 3B). Following TMT treatment, the protein level of mitochondrial COX4 increased in males, but not in females, both cortex and hippocampus (cortex: $p < 0.05$, hippocampus: $p < 0.001$) (Figs. 2, 3). These results indicate that TMT alters the expression of genes involved in mitochondrial biogenesis and that this change varies according to gender and brain region.

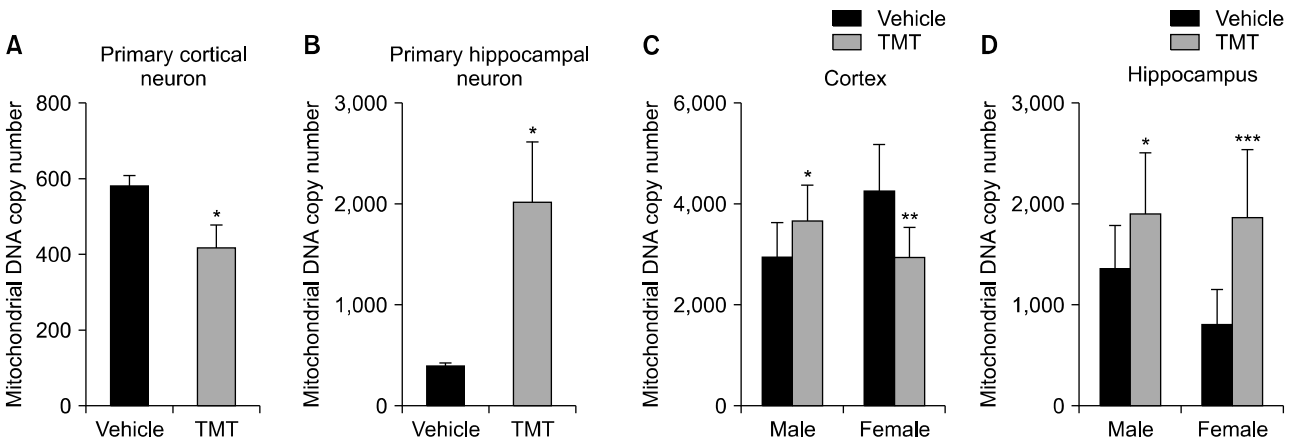

Fig. 1. The change of mitochondrial DNA copy number after trimethyltin (TMT) treatment in primary neuronal cultures and mice. (A, B) The change of mitochondrial DNA copy numbers after TMT treatment (5 μM) in primary cortical neuron (n = 3) (A) and primary hippocampal neuron (n = 3) (B). (C, D) The change of mitochondrial DNA copy numbers after TMT injection in cortices (male vehicle, n = 10; male TMT, n = 10; female vehicle, n = 9; female TMT, n = 10) (C) and hippocampi (male vehicle, n = 10; male TMT, n = 10; female vehicle, n = 9; female TMT, n = 10) (D) of male and female mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

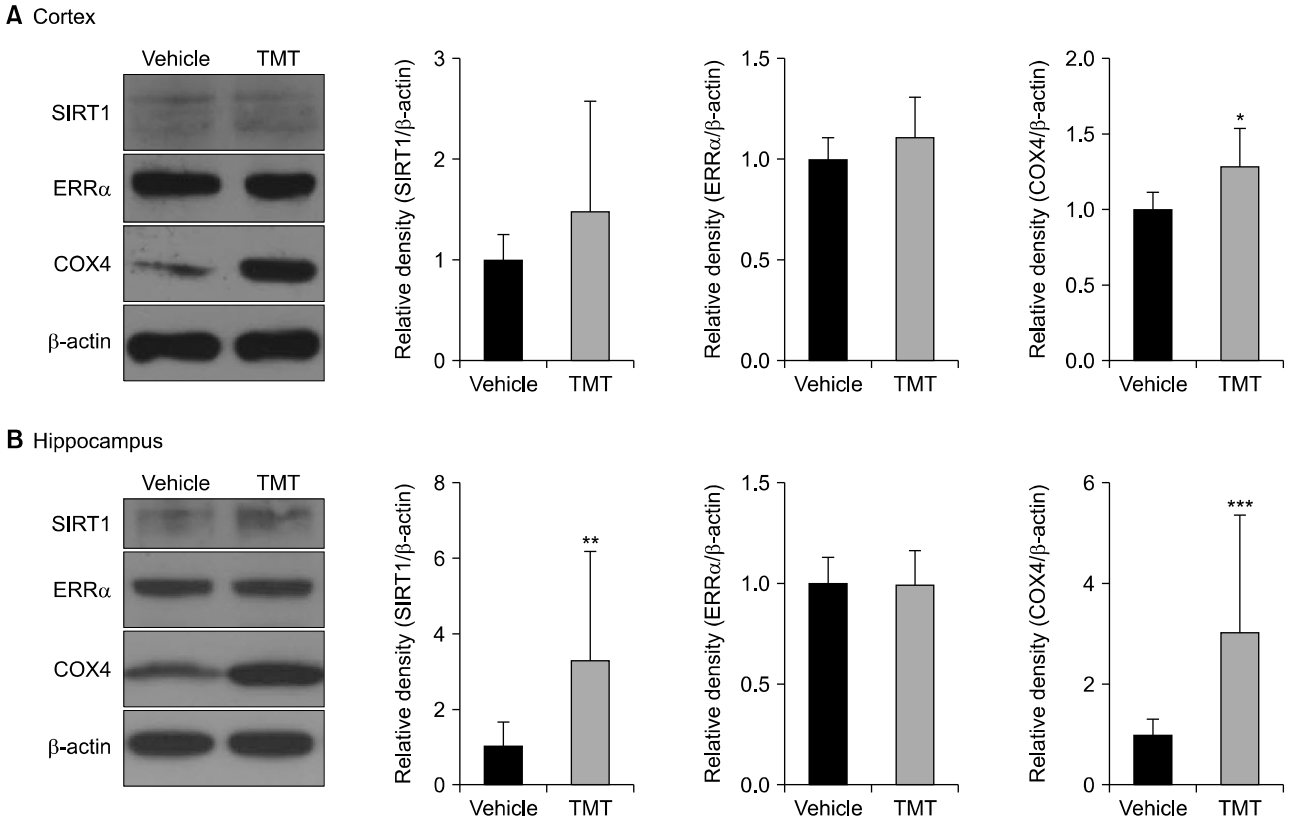

Fig. 2. Trimethyltin (TMT) treatment altered the protein level of mitochondrial biogenesis-related genes in brains of male mice. (A) (Left) Representative Western blots. (Right) Quantification of SIRT1 (vehicle, n = 8; TMT, n = 8), ERRα (vehicle, n = 8; TMT, n = 8), COX4 (vehicle, n = 8; TMT, n = 8) protein levels in the cortex. (B) (Left) Representative Western blots. (Right) Quantification of SIRT1 (vehicle, n = 16; TMT, n = 16), ERRα (vehicle, n = 8; TMT, n = 11), COX4 (vehicle, n = 15; TMT, n = 13) protein levels in the hippocampus.
SIRT1, sirtuin 1; ERRα, estrogen-related receptor alpha; COX4, cytochrome C oxidase subunit IV.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

DISCUSSION

Notably, mitochondrial biogenesis can increase in response to mitochondrial dysfunction owing to oxidative stress [17,18]. Previously, several studies have reported that oxidative stress-mediated apoptotic cell death occurs after TMT treatment [2,19,20], with numerous reports revealing that TMT toxicity varies with brain regions [21,22]. Moreover, another study showed that TMT demonstrates selective effects in the hippocampus and neocortex via stannin, located in the mitochondria [23]. In terms of metabolism and neurophysiology, primary neuronal cell cultures from different regions may present markedly different characteristics [24]. In the present study, the degree of alterations in mitochondrial biogenesis induced by TMT differed depending on the origin of the primary neurons. Furthermore, regional differences in the cortex and hippocampus of the female mouse brain, and sex differences in the cortex in terms of mtDNA copy numbers, were interesting findings. In our recent study, when we treated TMT to mice, the estradiol level significantly decreased only in female mice brains, and CYP1B1 level involved in estrogen metabolism increased only in female mice brains [25]. This difference in sex hormone changes is thought to involve the sex difference in the TMT effect. The mRNA expression of stannin, which is known to play an essential role in the neurotoxic effect of TMT, was found to be higher in the hippocampus than in the cortex [26]. In addition, as a limitation, we used the company-purchased primary neuron in this study, and we could not find any gender content in the product information. If we have gender information, we could have interpreted the sex difference in TMT in more detail. Further research is crucial to understand the underlying mechanisms that impact the inherent brain regional differences induced by TMT neurotoxicity.

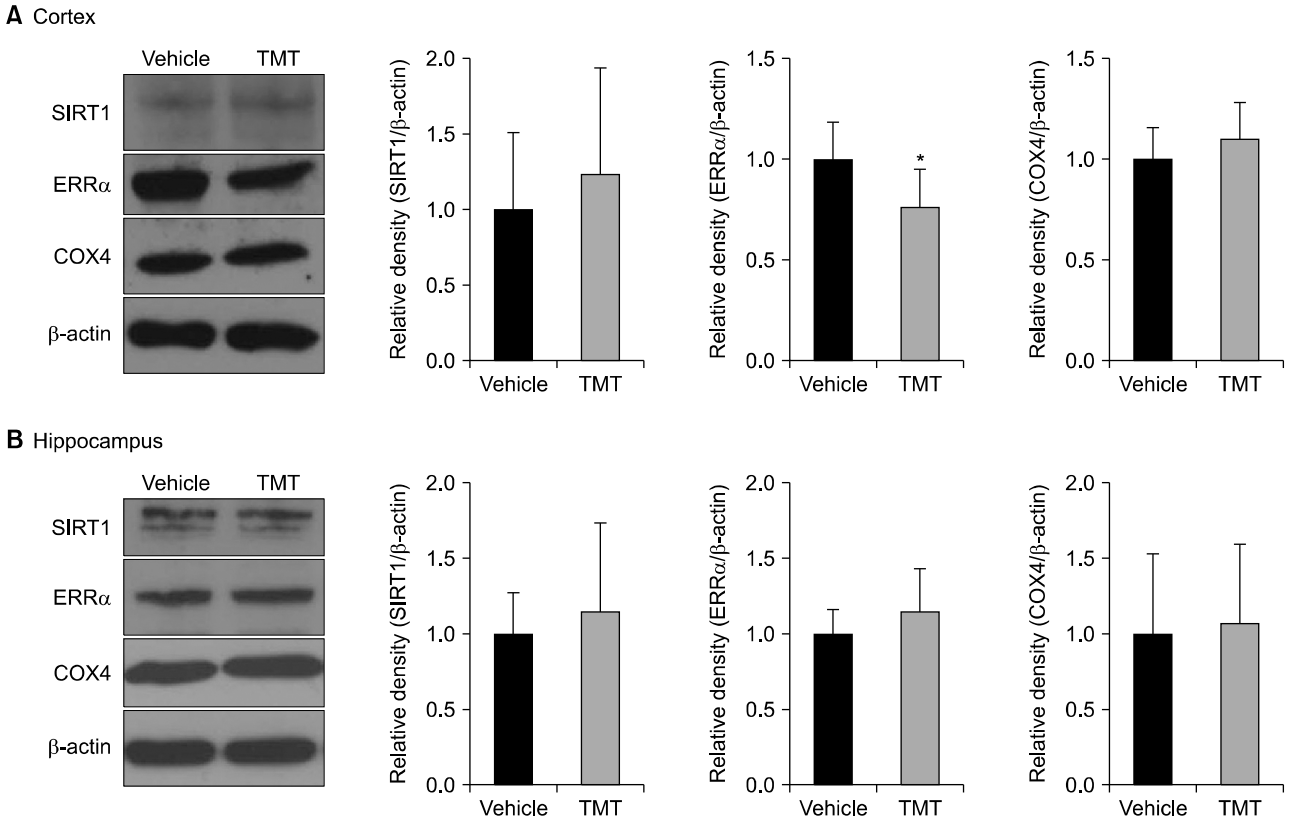

Fig. 3. Trimethyltin (TMT) treatment altered the protein level of mitochondrial biogenesis-related genes in brains of female mice. (A) (Left) Representative Western blots. (Right) Quantification of SIRT1 (vehicle, n = 8; TMT, n = 8), ERR$_\alpha$ (vehicle, n = 8; TMT, n = 8), COX4 (vehicle, n = 8; TMT, n = 8) protein levels in the cortex. (B) (Left) Representative Western blots. (Right) Quantification of SIRT1 (vehicle, n = 16; TMT, n = 15), ERRα (vehicle, n = 12; TMT, n = 12), COX4 (vehicle, n = 16; TMT, n = 15) protein levels in the hippocampus.
SIRT1, sirtuin 1; ERRα, estrogen-related receptor alpha; COX4, cytochrome C oxidase subunit IV.
*$p < 0.05$.

It is well known that mitochondria dysfunction, a significant source of oxidative stress, is a common pathology of several neurodegenerative diseases [27-29]. Reportedly, decreased mtDNA copy numbers have been observed in patients presenting neurodegenerative diseases [30-32]. Interestingly, the significance of mitochondrial dynamics in neuronal development has been outlined in the animal brain, and recent studies have reported morphological changes in mitochondria when neuronal stem cells differentiate in the developing and adult brains [33]. Mitochondrial biogenesis may represent an attempt to maintain a pre-existing aerobic set point in the presence of declining mitochondrial function. It is reasonable to consider that the increased brain mitochondrial mass could be compensatory in the young brain experiencing mitochondrial dysfunction [34].

Multiple factors intricately work together to regulate mitochondrial biogenesis [35,36] and SIRT1 and ERRα are two such well-known factors. SIRT1, the most widely investigated sirtuin, is known to demonstrate a significant effect on mitochondrial biogenesis, by regulating the acetylation of peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α and has presented protective effects in previous reports [37,38]. ERRα acts as a cofactor with PGC1α, affecting the transcription of several genes involved in mitochondrial functions [39,40]. Factors involved in mitochondrial biogenesis may demonstrate various changes in models related to neurodegenerative disease. The changes in SIRT1 level were not consistent in the results of studies using patients and animal models of various neurodegenerative diseases [41-43]. Furthermore, studies have documented a decrease in ERRα in neurodegenerative disease models [44,45]. Moreover, increased SIRT1 expression has shown neuroprotective effects in neurodegenerative disease models, and ERRα protein reportedly plays an important role in the pathology of

Alzheimer's disease [41,44].

In contrast to chronic neurodegenerative toxic effects, the altered protein expression patterns of ERRα and SIRT1 following acute TMT exposure differed between sexes and brain regions, in the present study. It was observed that the mitochondrial DNA copy number increased, and the expression of genes related to mitochondrial biogenesis increased in the mitochondria toxic propionic acid treated SH-SY5Y cells [46]. Though several drugs that cause neurotoxicity have been shown to decrease mitochondrial biogenesis-related gene expression [47,48], the mRNA expression of SIRT1 in the TMT-treated rat hippocampus shown a tendency to elevate compared to the control group in a previous study to investigate the effect of estrogen in rat brains treated with TMT [49]. After treatment with amyloid-beta soluble oligomer, it was observed that the SIRT1 protein level increased at 24 hours than 1 hour in PC12 cells, and it suggested an increase in SIRT1 level play a role in maintaining mitochondrial homeostasis [50]. Although the male mouse brain finally demonstrated increased COX-4 protein expression, the female brain showed a relative absence of differences in COX-4 protein expression after TMT exposure. Further studies are needed to clarify the mechanisms underlying regional and sex-specific differences in short-term mtDNA copy number alterations and the long-term effects on mitochondrial biogenesis related gene expression after TMT exposure.

In this study, we first demonstrated that short-term TMT treatment in early-juvenile mice induced region-specific and sex-specific alterations in mitochondrial biogenesis in the brain. To the best of our knowledge, the differences in mitochondrial biogenesis between the brains of male and female mice in the earlier life stage, first identified in our study, indicate that male and female brains have existing differences in metabolic characteristics prior to significant sexual maturation. Our results present a new perspective on neurological problems that can occur in men and women when accidentally exposed to environmental neurotoxins such as TMT at an early age.

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■ Conflicts of Interest

No potential conflict of interest relevant to this article was reported.Conflicts of Interest

■ Author Contributions

Conceptualization: Soon Ae Kim. Data acquisition: Jung Ho Lee, Eun Hye Jang. Formal analysis: Jung Ho Lee, Eun Hye Jang. Funding: Soon Ae Kim. Supervision: Soon Ae Kim. Writing—original draft: Soon Ae Kim, Jung Ho Lee, Eun Hye Jang. Writing—review & editing: Soon Ae Kim, Jung Ho Lee.

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