

Sex-specific Changes in Brain Estrogen Metabolism Induced by Acute Trimethyltin Exposure

JUNG HO LEE¹, SUNG-HEE CHO², EUN HYE JANG¹ and SOON AE KIM¹

¹Department of Pharmacology, School of Medicine, Eulji University, Daejeon, Republic of Korea;

²Chemical Analysis Center, Korea Research Institute of Chemical Technology (KRICT), Daejeon, Republic of Korea

Abstract. *Background/Aim:* In this study, we investigated sex-specific effects of acute exposure to trimethyltin, a known neurotoxicant on metabolic steroids. *Materials and Methods:* We administered intraperitoneally 2.3 mg/kg trimethyltin to 4-week-old male mice and measured the levels of metabolic steroids 24 h after treatment. We also measured mRNA and protein levels of cytochrome P450 1B1 using real-time polymerase chain reaction and western blotting. *Results:* Cortisol levels in the cortex increased in both sexes following acute trimethyltin exposure. The estradiol levels decreased, and the 4-hydroxyestradiol levels increased only in females. We also observed increased cytochrome P450 1B1 mRNA and protein levels only in the female cortex. *Conclusion:* Acute trimethyltin exposure induces distinct sex-specific metabolic changes in the brain before significant sexual maturation.

Trimethyltin (TMT), a tri-substituted organotin, is a neurotoxicant that causes neuronal degeneration in human's and animal's central nervous system by inducing oxidative stress and apoptosis (1). TMT causes neuronal loss in specific brain regions such as the dentate gyrus, olfactory bulb, anterior olfactory nucleus, and frontal cerebral cortex (2, 3). Exposure to high concentrations of TMT due to accident or experimental treatments induces characteristic behavioral changes in rodents (4, 5). There have been many studies on the effects of TMT on the brain of adult animals or neonatal development (5-7), but relatively few studies on the impact on juvenile mice. Since the brain continues to

develop during puberty, the adolescent brain is particularly vulnerable (8). TMT is likely to act as an environmental toxin in people of all ages, as low concentrations of TMT are observed even in people with no exposure experience (9). Therefore, it is crucial to know the effects and mechanisms of TMT on the adolescent brain.

Furthermore, sex differences are known to be involved in the incidence of brain diseases (10-13). To understand the differences between male and female brains causing these phenomena, a growing number of evidence suggests that both male and female models should be included in animal and cell experiments (14). Glucocorticoids and sex hormones are considered important factors for understanding sex differences in various physiological phenomena. Several reports have suggested that these hormones play a beneficial role in brain metabolism (15, 16) and disease risk (17-19). Since puberty sex hormones also have an organizational effect on the central nervous system; they can have long-term behavioral effects (20). However, sex differences in pubertal hormone metabolism following acute chemical exposure have been scarcely investigated.

In the present study, following TMT administration, we determined how TMT treatment affects the concentration and metabolism of metabolic steroids in female and male 4-week-old early juvenile mice brains.

Materials and Methods

Animal model. Three-week-old C57BL/6 mice were obtained from Samtako Bio Korea (Osan, Korea), and underwent adaptation in a room with controlled temperature (22±2°C), humidity (50±10%), and a 12 h light/dark cycle, with free access to water and food. After one week, mice were intraperitoneally administered either 2.3 mg/kg TMT diluted in normal saline (TMT group) or normal saline (control group). Both groups were administered 20 ml/kg solutions. After 24 h, mice were sacrificed using isoflurane (Hana Pharm, Seoul, Republic of Korea) anesthesia, and brains were excised and stored at -80°C. All procedures were conducted in accordance with the guidelines of the Eulji University Institutional Animal Care and Usage Committee (IACUC, document number: EUIACUC-17-12) and the ARRIVE guide for the care and use of laboratory animals.

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Correspondence to: Soon Ae Kim, MD, Ph.D., Department of Pharmacology, School of Medicine, Eulji University, 77, Gyeryong-ro 771beon-gil, Jung-gu, Daejeon 34824, Republic of Korea. Tel: +82 422591672, e-mail: sakim@eulji.ac.kr

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Western blotting. Total protein from the mouse brain cortex was isolated using the RIPA buffer (ATTO, Tokyo, Japan). Protein concentrations were measured using the bicinchoninic acid (BCA) assay. Briefly, 20 µg of cytosolic protein were separated on 10 % SDS-PAGE gels and transferred to nitrocellulose membranes (Pall, Port Washington, NY, USA). Next, the membranes were blocked with 5% non-fat milk in TBST buffer for 1 h at 4°C and incubated with primary anti-CYP1B1 antibody (Abcam, Cambridge, MA, USA) at 4°C overnight. The membranes were rinsed with TBST (Tris-buffered saline, 0.1% Tween 20) buffer, and incubated with horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h. After washing with TBST buffer, the membranes were incubated with West Femto Maximum Sensitivity Substrate (Thermo Fisher, Waltham, MA, USA). Protein expression levels were detected by exposure to an X-ray film (Agfa, Mortsel, Antwerp, Belgium), and analyzed using the ImageJ software (NIH, Bethesda, MD, USA).

Total RNA isolation and quantitative PCR. Total tissue DNA from mice cortices, was extracted using the miRNeasy mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized from isolated RNA using the RT2 first strand kit (Qiagen). Target gene amplification was performed using iQ™ SYBR® Green Supermix (Bio-rad, Hercules, CA, USA) in a CFX96™ Real-Time system (Bio-rad). Gene expression was normalized to GAPDH. GAPDH primer sequences were as follows: forward, 5'-GAGTCAACGGATTTGGT CGT-3'; reverse, 5'-GATCTCGCTCCTGGAAGATG-3'. CYP1B1 primer sequences were; forward, 5'-GAGGACTTTGATCCA GCCCG-3'; reverse, 5'-TGGAGATGAAGAGAAACAGAAGCA-3'. Relative mRNA expression was assessed using the $2^{-\Delta\Delta C_t}$ method.

Preparation of cortical tissue samples. Cerebral cortical samples were prepared as previously described (21). Briefly, 20 µl of the internal standard mixture was added to the sample (50 ng/ml) and then homogenized in 1 ml of methanol/acetic acid (99:1, v/v). After overnight extraction at 4°C, the samples were centrifuged at $6,652 \times g$ for 5 min, and pellets were extracted twice with 1 ml of MeOH/acetic acid (99:1, v/v). Next, the organic phases were combined and dried under a nitrogen stream. The samples were resuspended in 1 ml of MeOH/water (10:90, v/v). The mixture was extracted with Oasis PRiME HLB SPE cartridges (Waters Corporation, Milford, MA, USA). Then, the combined methanol eluates were evaporated under a nitrogen stream and dissolved in methanol (100 µl). Finally, 5 µl of this solution was injected into the liquid chromatography with tandem mass spectrometry (LC-MS/MS) system.

Metabolic profiling of steroids using LC-MS/MS. LC-MS/MS analysis was performed using an Acquity UPLC I-Class system (Waters Corporation), interfaced with a Xevo TQ-S micro tandem mass spectrometer (Waters Corporation) and an electrospray ionization (ESI) source. Chromatographic separation was achieved with an Acquity UPLC BEH C18 octadecylsilane column (2.1 mm \times 100 mm, 1.7 µm, Waters Corporation). For separation, the LC conditions were as follows: mobile phase A was 0.2 mM ammonium fluoride in H₂O, and mobile phase B was 0.2 mM ammonium fluoride in methanol. The gradient program (v/v) was performed at a flow rate of 400 µl/min and was initiated with 30% B (v/v), maintained at 30% B (v/v) for 5 min, increased to 50% B (v/v) at 15 min, maintained at 50% B (v/v) for 5 min, increased to 55% B

(v/v) at 23 min, increased to 80% B (v/v) at 27 min, and then maintained at 80% B (v/v) for 3 min. Then, the column was re-equilibrated for 3 min with 30% B (v/v). The source and operating parameters were optimized as follows: capillary voltage of 3 kV (ESI+) and 4 kV (ESI-); cone gas flow of 30 l/h; desolvation temperature of 450°C; desolvation gas flow of 800 l/h. Quantitative analysis was performed in the multiple reaction monitoring (MRM) mode. Data acquisition was performed using the MassLynx software (V4.1, Waters Corporation).

Statistical analysis. All statistical analyses were conducted using IBM SPSS Statistics for Windows (version 20, IBM Corporation, Armonk, NY, USA). Quantitative results are expressed as mean \pm standard deviation, and groups were compared using Dunnett's test and unpaired *t*-test. Differences were considered statistically significant at a *p*-value less than 0.05 (*p*<0.05). Significant *p*-value data are denoted in the graphs as follows: **p*<0.05, ***p*<0.01, and ****p*<0.001.

Results

Alterations in metabolic steroid levels in the cerebral cortex of acute TMT-treated mice. To investigate the profile of metabolic steroids in male and female brains following TMT treatment, we performed an LC-MS/MS analysis. As expected, there was no significant difference in estradiol levels between 4-week-old female and male mice before TMT treatment. After TMT treatment, cortisol levels increased in both male and female brains (Figure 1A). In the male mouse brain, there was no change in estradiol levels after TMT administration (Figure 1B). The estradiol levels decreased, and the estradiol metabolite, 4-hydroxyestradiol levels increased in the female brain after TMT treatment (Figure 1C).

Sex-specific change in CYP1B1 mRNA and protein levels in the cerebral cortex of acute TMT-treated mice. We measured mRNA and protein levels of cytochrome P450 1B1 (CYP1B1), an enzyme that metabolizes estradiol using real-time PCR and western blotting. Interestingly, increased CYP1B1 mRNA levels were observed only in the female cortex after TMT treatment (Figure 2A). Furthermore, it was confirmed by western blot analysis that CYP1B1 protein levels were increased only in the TMT treated female cortex (Figure 2B).

Discussion

It is known that oxidative stress is associated with the induction of neurosteroid biosynthesis in the human brain, and TMT induces oxidative stress by mitochondrial dysfunction (22). Following TMT treatment, the cortisol levels, a representative stress hormone, increased in the brains of both male and female mice in this study. High cortisol levels have been reported to be associated with low

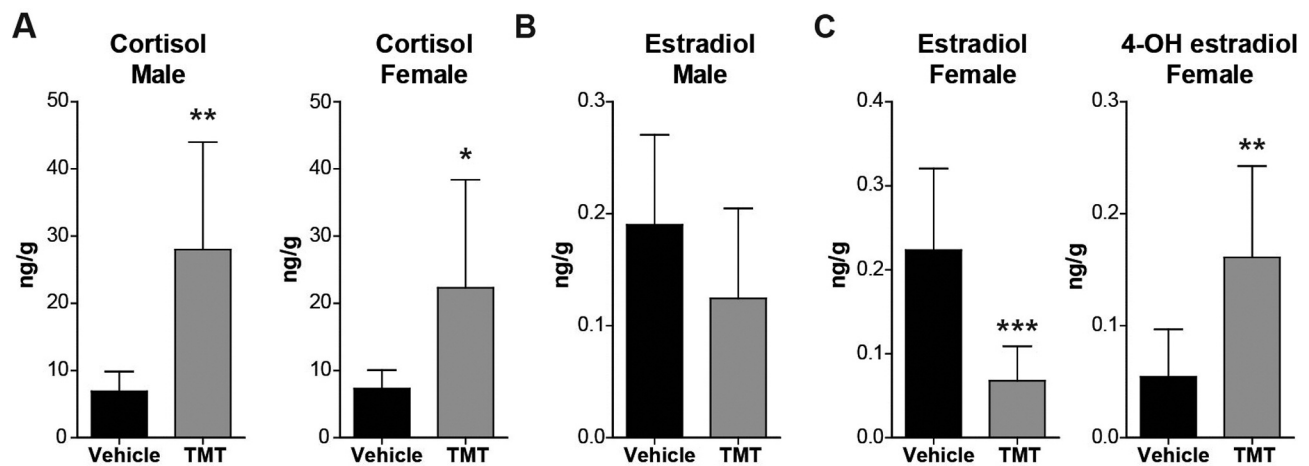


Figure 1. Changes in brain metabolic steroid levels in male and female mice brains. A. Cortisol levels in male (left, $n=10$) and female ($n=9$) mice brains. B. Estradiol levels in male (left, $n=10$). C. Estradiol (left, $n=9$) and 4-OH estradiol levels (right, vehicle, $n=9$, TMT, $n=10$) in female mice brains. Steroid levels were measured by LC-MS/MS analysis. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

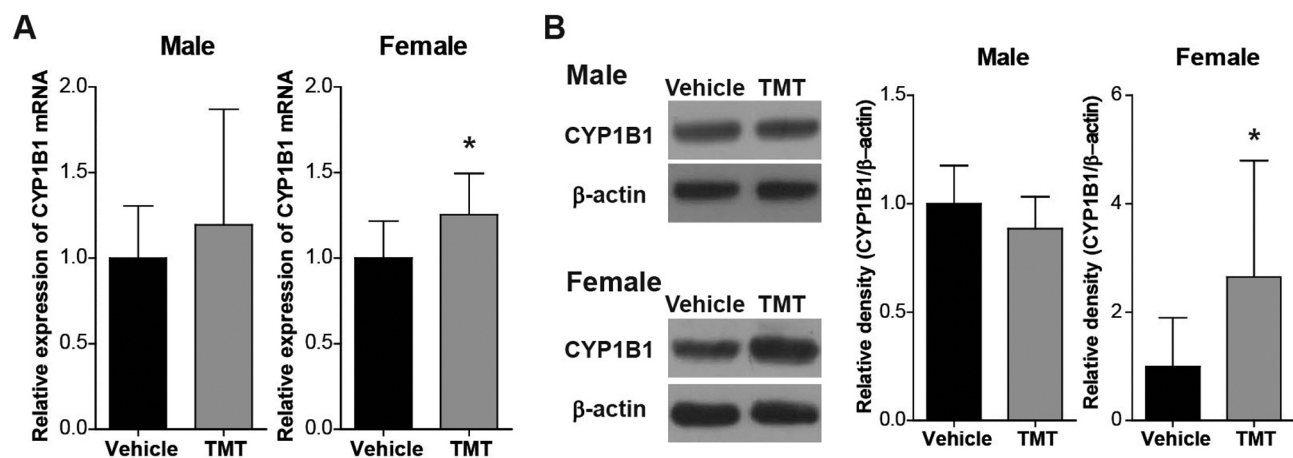


Figure 2. Changes in *CYP1B1* expression in male and female mice brains. A. Relative expressions of *CYP1B1* mRNA measured by RT-PCR in male (left, $n=10$) and female (right, $n=8$) mice brains. B. Representative western blots (left). Quantification of *CYP1B1* protein levels in male ($n=$) and female ($n=8$) mice brains (right). * $p<0.05$.

brain volume, memory impairment, and corticosterone treatment as well as changes in GABA uptake in the hippocampus, suggesting a mechanism for hormonal modulation of brain function (23, 24).

Estrogen is the primary female hormone, demonstrating pleiotropic effects on non-reproductive organs. Estradiol, a predominant and potent form of estrogen, plays an essential role in brain development and proper brain functioning, including mood and various cognitive activities (25). Several previous studies have shown the neuroprotective effects of estradiol in neurodegenerative diseases (26, 27). To the best of our knowledge, only two studies have investigated the effects of estradiol in TMT-induced animal brain damage. In

one study, pharmacological administration of estradiol in TMT-treated 2-month-old rats upregulated genes involved in neuroprotection and synaptogenesis (28). In the second study, pretreatment with estradiol reduced neuronal death in TMT-treated neonatal rats (29). It has recently been suggested that 4-hydroxyestrone can protect neuronal cells from oxidative damage (30). In this study, we observed increased 4-hydroxyestrone levels in TMT treated females, which may be a neuroprotective *in vivo* homeostatic response.

Notably, there are numerous differences in the brains of females and males regarding metabolism. Using aged mice, Zhao *et al.* have reported altered expression of genes involved in metabolism between female and male rats (31). Our study

revealed, for the first time, that the levels of endogenous estradiol and its metabolite were altered in the female brain after TMT exposure. Additionally, TMT exposure induced CYP1B1 expression only in female mice. Our data suggest that it is crucial to evaluate estrogen metabolism to elucidate the mechanism of TMT toxicity concerning sex differences. Reportedly, CYP1B1 expression is regulated by the aryl hydrocarbon receptor (AhR) (32), which is a ligand-activated transcription factor that controls gene expression induced by xenobiotics (33). Increased 4-hydroxyestradiol, an estradiol metabolite produced *via* CYP1B1, may be induced by the AhR pathway *via* TMT acting as a xenobiotic (34, 35). Moreover, the AhR pathway has been reported to cross-talk with nuclear receptor pathways and be involved in neuronal progenitor cell expansion and the sex-dependent differentiation of GABAergic neurons in the hippocampus (36). As studies investigating the effects of 4-hydroxyestradiol and the mechanism of sex-dependent CYP1B1 induction are lacking, further research is warranted, especially regarding sex-specific differences concerning steroid metabolism in the brain.

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

Conflicts of Interest

The Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' Contributions

Jung Ho Lee: data analysis, paper writing. Eun Hye Jang: cell culture, animal treatment, biochemical experiments, manuscript writing. Sung-Hee Cho: LC-MS/MS, data analysis, manuscript revision, made a critical contribution to the discussion. Soon Ae Kim: overall design, manuscript revision.

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