RESEARCH PAPER

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Diverse changes in myelin protein expression in rat brain after perinatal methadone exposure

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The national incidence of neonatal abstinence syndrome has dramatically increased over the last decade due to an increase in antenatal opioid exposure. Recent human and animal studies suggest that antenatal opioid exposure impacts the developing brain. The purpose of this study is to evaluate the effects of perinatal methadone exposure on myelination in multiple regions in the developing rat brain. Pregnant Sprague-Dawley rats were randomly assigned into three experimental groups and subsequently exposed to drinking water alone or drinking water containing methadone from 7 days post coitum through day 7 or through day 19 after delivery. Two male neonatal rats were randomly selected from each litter and terminated at day 19. The cerebral cortex, hippocampus, cerebellum, and brainstem were dissected and analyzed for three myelin specific proteins – CNP, PLP, and MBP – by Western blot analysis. All pups with exposure to methadone demonstrated decreased expression of CNP, PLP, and MBP in the cerebral cortex and hippocampus. In the cerebellum, PLP expression was down-regulated without apparent alteration of CNP and MBP expression. PLP and MBP expression, but not CNP expression, were significantly inhibited in the brainstem. Compared to the pups with postnatal methadone exposure via maternal milk through day 7, partial recovery of CNP and PLP expression only occurred in the cerebral cortices of the pups exposed through day 19. The findings show that antenatal opioid exposure in rat pups is associated with regionally-specific alterations in brain myelination that diversely affects myelin proteins.

Key words: neonatal abstinence syndrome, varied expression, myelin, methadone, antenatal opioid exposure, brain

INTRODUCTION

The medical and non-medical use of opioids has increased exponentially over the last decade in women of childbearing age (Centers for Disease Control and Prevention, 2016). An increase in antenatal exposure to opioids has resulted in a greater than 5-fold rise in the national incidence of neonatal abstinence syndrome (NAS) (Winkelman et al., 2018). A retrospective analysis of infants covered by Medicaid across the United States demonstrated an escalation in incidence of NAS from 2.8/1000 live births in 2004 to 14.4/1000 live births in 2014 (Winkelman et al., 2018).

The developmental impact of antenatal opioid exposure remains poorly delineated, but recent human and animal studies provide insight into the influence of antenatal exposure on the structure of the developing brain. Walhovd et al. (2007) utilized MRI imaging to demonstrate that infants with exposure to illicit psychotropic polysubstances, including heroin during pregnancy, had smaller brain volumes, including white matter volumes, than those not exposed. Additional studies employing

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MRI imaging found that infants with antenatal exposure to opioids (e.g., medication assisted treatment (MAT) with methadone and buprenorphine and illicit opioids such as heroin) and other psychotropic drugs had decreased whole brain volumes when compared to unexposed infants (Yuan et al., 2014) and that altered maturation of cerebral connective tract development may accompany *in utero* methadone exposure (Walhovd et al., 2012).

Rat model data remains inconclusive regarding methadone effects on myelination. Rat pups with antenatal exposure to methadone via an osmotic minipump implanted in the pregnant dam demonstrated an up-regulation of myelin production and altered structure (Vestal-Laborde et al., 2014). Alternatively, rat pups with antenatal exposure to methadone via drinking water demonstrated decreased expression of myelin-specific proteins (Gourevitch et al., 2016).

Detoxification during pregnancy has been associated with high rates of relapse, extreme maternal stress, and possible harm to the fetus. Therefore, MAT with methadone or buprenorphine, is recommended by the American College of Obstetrics and Gynecology (ACOG) for treatment of women with opioid addiction during pregnancy (ACOG Committee, 2012). The purpose of this pilot study was to evaluate the effects of passive antenatal and passive postnatal methadone exposure via maternal milk on myelin development in various regions of the rat brain.

METHODS

Animals

Twelve-week-old male and female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed with food and water available ad libitum in an animal facility maintained at a temperature (22±2°C) and in a light-controlled environment with an alternating 12-hour light/dark cycle. All procedures were supervised by the University of Louisville Research Resources Center and an Association for Assessment and Accreditation of Laboratory Animal Care approved facility and performed in accordance with the guidelines of the Animal Care and Use Committee of University of Louisville School of Medicine and NIH requirements for care and use of laboratory animals.

Experimental groups and perinatal methadone (MTD) exposure model

Female rats were mated with a male rat and confirmed for insemination. Neural development and myelination of the rat brain at postnatal day 7 (p7) is roughly equal developmentally to a full-term human baby (Clancy et al., 2001; 2007). Then, nine pregnant rat dams were randomly assigned into three experimental groups (Fig. 1): dams exposed to drinking water alone (Control); dams exposed to drinking water containing MTD from 7 days post-coitum (E7) to P7 (MTD E7-P7). To avoid abnormal nursing behaviors in the dams due to MTD withdrawal, the rat dams with MTD drinking water were replaced by lactating rats with drinking water alone; and dams exposed to drinking water containing MTD from E7 to P19 (MTD E7-P19). Ten neonatal pups each from litter were retained at birth to eliminate variation in growth due to the differences in litter size. For rat pups born to dams in different MTD-exposed groups, MTD exposure was maintained via placenta antenatally and via breast milk postnatally.

During pregnancy, an increase in human daily oral dose of MAT may be required due to rapid clearance of MTD. Since a dose of 3 mg/kg body weight per day has not been shown to disrupt pregnancy in rats (Pierce et al., 1992; Daly et al., 2012), assuming a body weight of 400 g, the pregnant dam was provide with 0.2 ml MTD (150 mg/ml) diluted in 1 liter drinking water based on daily water intake ad libitum (40 ml) (Atherton et al., 1982). This dose is roughly equivalent to the maximum day 1 dose of MAT (40 mg) based on an average weight of 75 kg for American women following the extrapolation of dose between species published previously (Nair and Jacob, 2016), and did not result in global growth retardation in MTD-exposed pups until P19.

Tissue processing

Rodent white matter development exhibits a sexual dimorphism (Yang et al., 2008; Cerghet et al., 2009) probably due to different levels of steroid hormones (Jung-Testas and Baulieu, 1998). The expression of myelin-specific proteins, including 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), proteolipid protein (PLP),

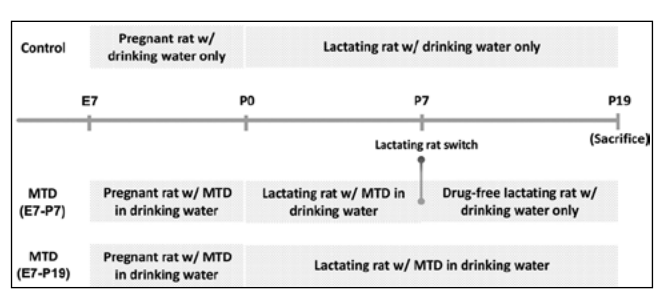

Fig. 1. Experimental workflow for perinatal MTD exposures and group assignment.

and myelin basic protein (MBP), showed increased elevation in males compared with females independent of strain and species (Cerghet et al., 2006). Thus, six male rats (two rats per litter) in each experimental group were anesthetized with isoflurane and decapitated at P19, as the maximal rate of myelin accumulation occurs at this age (Doretto et al., 2011). The whole brain was rapidly removed from the skull into a chamber perfused with chilled artificial cerebrospinal fluid containing (in mM) 128 NaCl, 3 KCl, 1.4 CaCl2, 1 MgSO4, 21 NaHCO3, 0.5 NaH2PO4, and 30 glucose, equilibrated with 95% O2-5% CO2. Four regions (cortex, hippocampus, cerebellum, and brain stem) were dissected out on ice for protein preparation.

Western blots

Protein samples were prepared in CelLytic™ MT Cell Lysis Reagent (Sigma-Aldrich, St. Louis, MO) containing the complete protease inhibitors (Roche, Indianapolis, IN) on ice. Western blots were performed as described previously (Cai et al., 2012; Wang et al., 2018). Equivalent amounts of total protein (30 µg/lane) were separated using 10% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) and then electrometrically transferred onto nitrocellulose membranes (Protan BA83, Midwest Scientific, Valley Park, MO). After blocking in 5% fat-free milk for one hour to reduce non-specific antibody binding, the membranes were incubated overnight at 4°C with the following primary antibodies: mouse anti-PLP (1:2000; Calbiochem), rabbit anti-MBP (1:3000; Millipore), mouse anti-CNPase (1:4000; Millipore), and mouse anti-β-actin (1:5000; Sigma-Aldrich). After three washes, the membranes were incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3000; Santa Cruz) for one hour. The immunoblots were visualized using ECL western blotting detection reagent (Pierce, Grand Island, NY). The optical density (OD) of the protein bands was analyzed using the BIO-RAD ChemiDocTM Touch Imaging System (BIO-RAD, Hercules, CA). The ODs for specific proteins were normalized to the corresponding β-actin levels, and these values were expressed as the ratio relative to the control. An investigator who was masked to the group assignment performed the analysis.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Comparisons between three experimental groups were conducted using one-way analysis of variance (ANOVA) followed by *post-hoc* Tukey's test using GraphPad Prism 5.0 (GraphPad Software, San Diego California USA). The significance level was $p<0.05$.

RESULTS

Opposite responses in myelin protein synthesis after perinatal MTD exposure in immature and mature oligodendrocytes in brain stem

The expression levels of myelin specific proteins (CNP, PLP, and MBP) were measured and compared to those of the control group to identify alterations in myelin development after MTD exposure. CNP is localized in immature oligodendrocytes, while PLP and MBP are synthesized in mature oligodendrocytes. After dams were exposed to MTD from either E7–P7 or

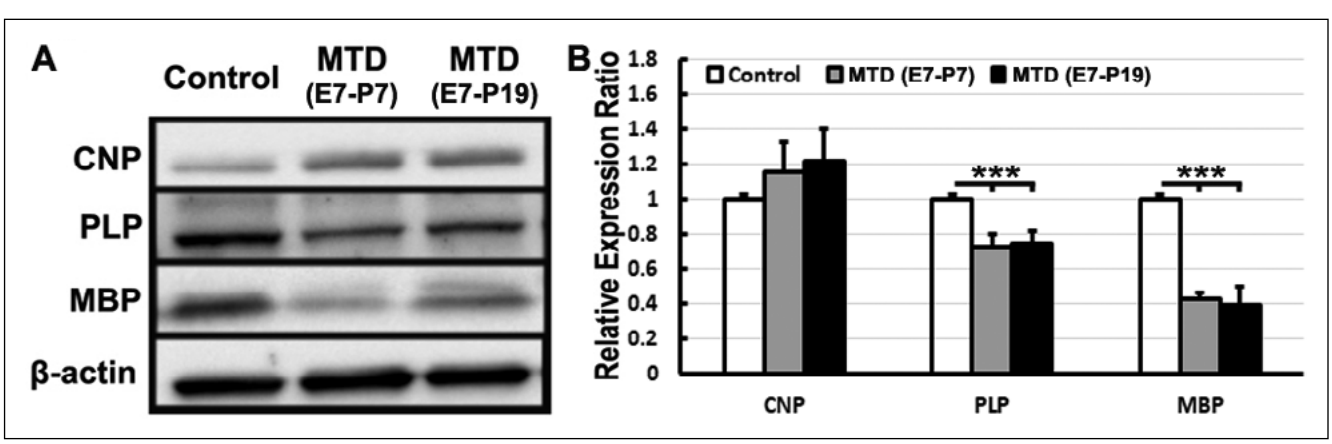

Fig. 2. Representative Western blots (A) and statistical analysis (B) of myelin specific proteins CNP, PLP, and MBP in brainstems dissected from control and perinatal MTD-exposed rat pups. The data were presented as mean ± SD values and analyzed by one-way ANOVA followed by *post-hoc* Tukey's test. * represents $p<0.05$ vs. control; n=6 pups randomly selected from 3 dams per group, respectively.

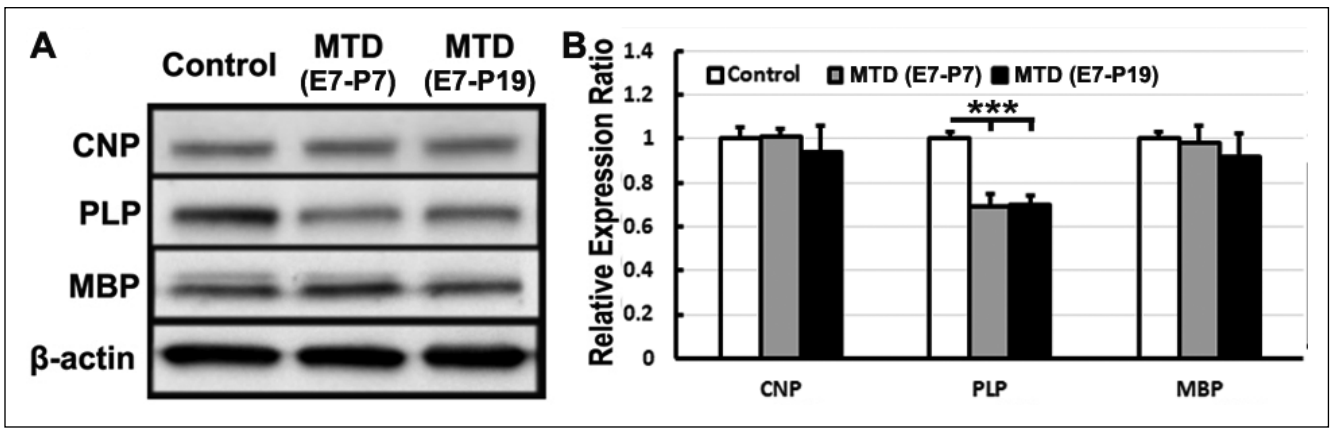

Fig. 3. Representative Western blots (A) and statistical analysis (B) of myelin specific proteins CNP, PLP, and MBP in cerebella dissected from control and perinatal MTD-exposed rat pups. The data were presented as mean ± SD values and analyzed by one-way ANOVA followed by *post-hoc* Tukey's test. *** represents $p<0.001$ vs. control; n=6 pups randomly selected by 2 pups per litter from 3 dams for each group, respectively.

E7–P19, pups showed a significant decrease in PLP and MBP expression ($p<0.001$ *vs.* control, n=6) but an increasing trend in the expression of CNP in brainstem ($p=0.066$ *vs.* control, n=6). However, no statistical difference was observed between the two MTD exposure groups (Fig. 2).

Suppression of PLP expression after perinatal MTD exposure in the cerebellum

In the cerebellum, significant decreases in PLP expression were observed in both groups of perinatally MTD-exposed pups (E7–P7 and E7–P19) when compared to controls ($p<0.001$ *vs.* control, n=6). PLP expression in both groups of MTD-exposed pups was not significantly different. Surprisingly, differences in the expression levels of MBP and CNP for all three groups did not reach significance ($p>0.05$ *vs.* control, n=6) (Fig. 3).

Marked reduction of expression of myelin proteins in cerebral cortex and hippocampus after perinatal exposure to MTD

As shown in Fig. 4, expression levels of all three myelin proteins were significantly decreased in cerebral cortices after perinatal MTD exposures from either E7–P7 or E7–P19 (CNP and PLP: $p<0.001$ *vs.* control; MBP: $p<0.05$ *vs.* control; n=6). Moreover, CNP and PLP synthesis seemed to be more vulnerable to perinatal MTD exposures than MBP. Similarly, dramatic decreases of the three myelin proteins were observed in the hippocampus after perinatal MTD exposure (Fig. 5, $p<0.001$,

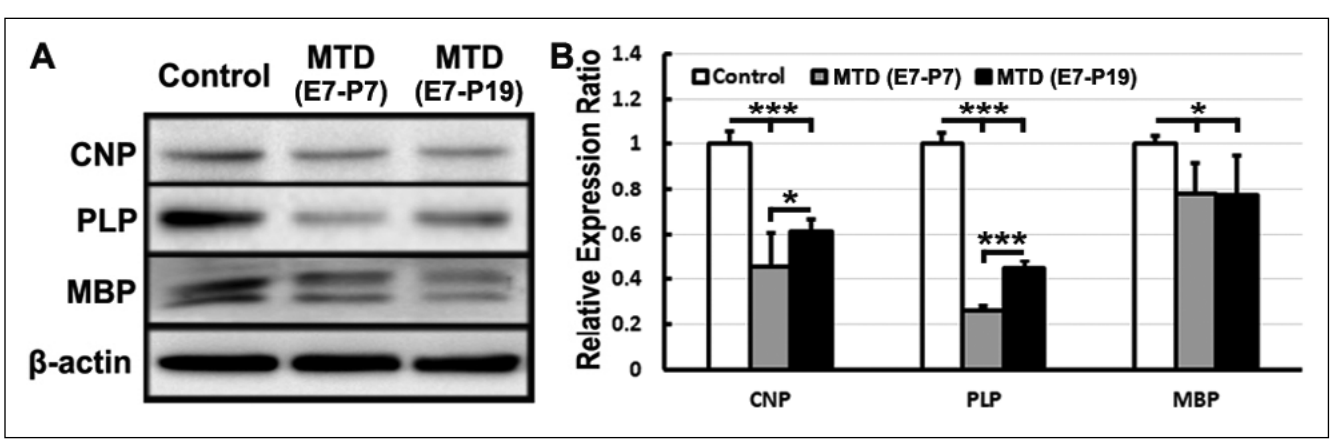

Fig. 4. Representative Western blots (A) and statistical analysis (B) of myelin specific proteins CNP, PLP, and MBP in cerebral cortices dissected from control and perinatal MTD-exposed rat pups. The data were presented as mean ± SD values and analyzed by one-way ANOVA followed by *post-hoc* Tukey's test. * represents $p<0.05$, *** represents $p<0.001$ vs. control or MTD (E7-P7); n=6 pups randomly selected by 2 pups per litter from 3 dams for each group, respectively.

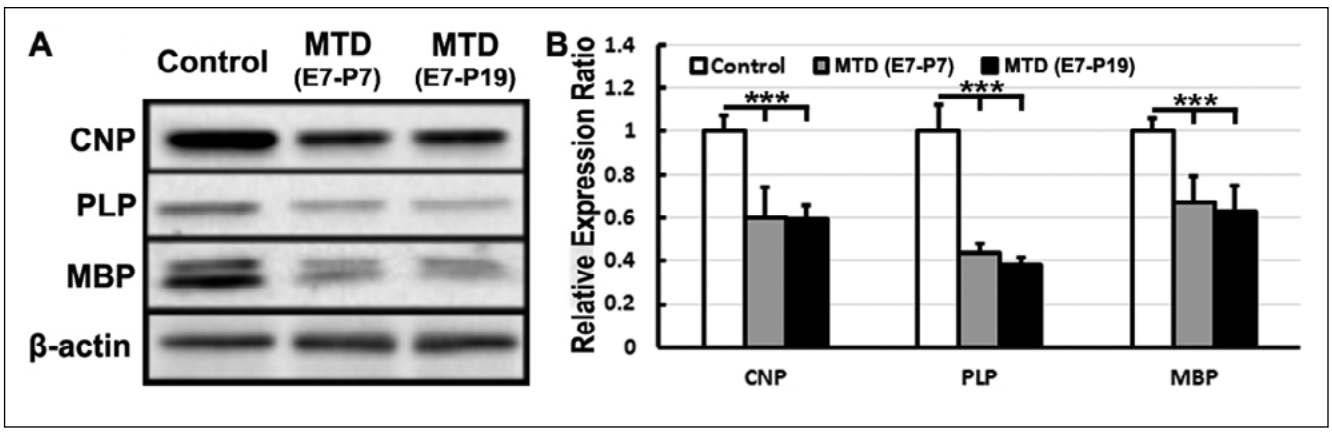

Fig. 5. Representative Western blots (A) and statistical analysis (B) of myelin specific proteins CNP, PLP, and MBP in hippocampi dissected from control and perinatal MTD-exposed rat pups. The data were presented as mean ± SD values and analyzed by one-way ANOVA followed by *post-hoc* Tukey's test. *** represents $p<0.001$ *vs* control; n=6 pups randomly selected by 2 pups per litter from 3 dams for each group, respectively.

n=6). Taken together, perinatal MTD exposure induced severe impairments of myelin protein synthesis in regions of cerebral cortex and hippocampus.

DISCUSSION

MTD is the gold standard for treatment of opioid dependency during pregnancy (Farid et al., 2008). Treatment with MTD, a full opioid agonist, during pregnancy has been shown to improve multiple outcomes including compliance with obstetrical care, increased birthweights, decreased neonatal death, and maintenance of custody with the biological parents at hospital discharge. Moderate to severe NAS is common in infants with antenatal MTD exposure. Delineating the physiologic changes in the developing brain after antenatal exposure provides a critical key to understanding long-term developmental outcomes in this vulnerable population.

The human brain undergoes rapid development in the first years of life, which includes accelerated myelination from birth until three years of age. Myelin development then continues throughout the first decade of life. Distinct brain regions vary in their rate of myelin maturation (Carmody et al., 2004). Walhovd et al. (2012) demonstrated delayed and altered neural tract development using MRI with diffusion tensor imaging in infants with antenatal exposure to methadone, which may underlie the increased risk for cognitive and behavioral difficulties in children. Gourevich et al. (2016), recently demonstrated results similar to ours with an overall trend of hypomyelination in brain stem when neonatal rat pups were exposed to MTD *in utero* and during the postnatal period, delivered by drinking water (3 mg/kg/day). Our study demonstrated variable expression of immature (CNP) and mature (PLP and MBP) myelin proteins among the four brain regions of interest. In the brain stem, an increasing trend in CNP expression and significant decrease in PLP and MBP expression suggest an impairment of oligodendrocyte differentiation and/or myelin formation with an impact on immature oligodendrocytes after MTD exposure. Since μ-opioid receptor activation stimulates oligodendrocyte proliferation (Knapp and Hauser, 1996; Vestal-Laborde et al., 2014), an increasing trend of CNP expression may reflect an increase in immature oligodendrocytes undergoing differentiation. In the cerebellum, however, a significant decrease was only detected in PLP expression while CNP and MBP did not demonstrate differences when compared to the control group. In the cortex and hippocampus, expression levels of CNP, PLP, and MBP were all significantly lower in MTD-exposed groups, indicating impairments of both immature and mature oligodendrocytes. Our findings show that passive antenatal exposure to MTD has variable effects on myelination in different regions of the developing rat brain. Of note, compared to those pups exposed to MTD transferred through maternal breast milk for seven days, no difference or partial recovery in the expression of myelin markers occurred in pups exposed to MTD for 19 days after birth, which suggests that *in utero* exposure to MTD may primarily contribute to myelin impairment whereas the impact of postnatal MTD exposure via breast milk is probably limited.

The long-term developmental consequences of perinatal opioid exposure remain largely unknown due to a lack of large longitudinal trials in this vulnerable population. A retrospective trial in 2018 found that children who were diagnosed and treated for NAS as infants demonstrated lower scores on the cognitive, language, and motor subscales of the Bayley Scales

of Infant Development, 3rd Edition at two years of age when compared to national norms (Merhar et al., 2018). A recent Australian study (n=2,236) linked a discharge diagnosis of NAS with standardized test scores and found that the diagnosis of NAS was associated with lower mean standardized test scores in the 3rd, 5th, and 7th grades and that the deficit was progressive (Oei et al., 2017). The mechanism for the developmental delays in children exposed to opioids *in utero* and in the postnatal period is not well delineated, although altered myelination has been proposed as a potential etiology (Vestal-Laborde et al., 2014). Our study shows that passive MTD exposure *in utero* and in the postnatal period through maternal breast milk is accompanied by hypomyelination in cerebral cortex, hippocampus, brainstem, and cerebellum in rat pups, which raises concern for similar effects in exposed infants.

There are several limitations to our study. This was a pilot study with a small sample size, hence the overall power to detect statistical significances is diminished. MTD dosage was based on prior accepted studies; however, translating drug dosage from rats to humans is difficult given pharmacokinetic and drug metabolism differences. Lastly, the specific endpoints we used have been cited as approximations of rat to human myelin maturity. Closer review of the literature demonstrates that opinions vary.

In conclusion, our study supports the conjecture that passive exposure to MTD during the perinatal period is accompanied by altered CNS development. Given the critical brain development that occurs from the time of conception through the first three years of life, altered myelination may contribute to neurodevelopmental impairment. The impact of this significant public health concern deserves further study.

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NM, LD and JC designed the study, interpreted the data, and coordinated the project. NM performed the animal treatments. NM, RJ and JC collected brain tissues. RO, TC, and HO conducted Western blots, data collection, and statistical analysis. The manuscript was written by RO and TC, revised by LD and JC. The submitted version was approved by all authors.

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