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Exposure to nonylphenol in early life increases pro-inflammatory cytokines in the prefrontal cortex: Involvement of gut-brain communication

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ABSTRACT

A growing body of evidence indicates that exposure to nonylphenol (NP), a typical persistent organic pollutant (POP), in early life results in the impairment of the central nervous system (CNS), but the underlying mechanism still remains to be elucidated. High levels of pro-inflammatory cytokines in the brain have been implicated in the CNS damages. The animal model of exposure to NP in early life was established by maternal gavage during the pregnancy and lactation in the present study. We found that exposure to NP in early life increased the levels of pro-inflammatory cytokines in the rat prefrontal cortex. Interestingly, the levels of pro-inflammatory cytokines in the intestine as well as in the serum were also increased by NP exposure. Furthermore, the increased permeability of intestinal barrier and blood-brain barrier (BBB), two critical barriers in the gut to brain communication, was observed in the rats exposed to NP in early lives. The decreased expression of zonula occludens-1 (ZO-1) and claudin-1 (CLDN-1), tight junction proteins (TJs) that responsible for maintaining the permeability of intestinal barrier and BBB, was found, which may underlie these increases in permeability. Taken together, these results suggested that the disturbed gut-brain communication may contribute to the increased levels of pro-inflammatory cytokines in the prefrontal cortex caused by NP exposure in early life.

Abbreviations:

BBB	blood-brain barrier
BCSFB	blood-cerebrospinal fluid barrier
CLDN-1	claudin-1
CNS	central nervous system
DAPI	4′,6-diamidino-2-phenylindole
ELISA	enzyme-linked immunosorbent assay
FITC-dextran	Fluorescein isothiocyanate-dextran
IL-1β	interleukin-1β
IL-6	interleukin-6
PND	postnatal day
POP	persistent organic pollutant
SEM	standard error of mean
TBST	Tris-buffered saline with Tween 20
TJs	tight junction proteins
TNF-α	tumor necrosis factor-α
VPA	valproic acid
ZO-1	zonula occludens-1

1. Introduction

Nonylphenol (NP) is a widely used material for the industrial production of surfactants, cleaners, wetting agents, and so on. It also ubiquitously exists in household toiletries, paints, cosmetics, and pesticides [1]. NP is released into varied environmental media, such as sediment, river water, and soil, mainly through the discharge of industrial and municipal wastewater [2]. NP is quite stable in the environmental media and considered as a typical persistent organic pollutant

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(POP), and it is largely absorbed and bio-accumulated by living organisms. Humans may be exposed to higher concentrations of NP through the food chain or seriously polluted drinking water [3]. Notably, NP has been detected in various human samples, such as breast milk, urine, adipose tissue, and fetal cord blood [4,5].

Exposure to NP is believed to impair the central nervous system (CNS) and cause cognition, attention, and motor dysfunction [6,7]. The positively linear correlations between NP concentrations in brain regions and poor behavioral performances in Morris water maze (MWM) and elevated plus maze were found in rats orally exposed to NP [8]. Besides, oral exposure to NP at low concentration was shown to impair the memory performance of rats in appetite-motivated maze test [9]. In another study, step-down avoidance test was conducted in rats subjected to maternal NP exposure, and the impairment of learning and memory ability was observed [10]. Gestation and lactation are two critical periods of neurodevelopment, and damages to the CNS during these periods may seriously affect the cognitive function, linguistic performance, intelligence, motor, and social function [11,12]. The fetuses and infants could be exposed to NP via transplacental absorption or breastfeeding during these periods. Their brains were quite vulnerable to NP neurotoxicity partially because of the immaturity of the blood-brain barrier (BBB). Consistent with this, it has been shown that exposure to NP during these critical periods of brain development irreversibly harmed learning and memory functions of offspring rats in adolescence or adulthood [6,7]. However, the potential mechanisms underlying these impairments of the CNS remain unclear.

It is well known that the abnormal increase in pro-inflammatory cytokines causes damages to the CNS, and greatly contributes to the pathogenesis of varied neurodevelopmental disorders [13,14]. Interestingly, intestinal disorders are often found in the patients of neurodevelopmental disability. Moreover, the elevated levels of pro-inflammatory cytokines in the intestine have been linked to the etiology of neurodevelopmental diseases [15]. The gut-derived pro-inflammatory cytokines are released into blood circulation under some pathological conditions, which lead to the abnormal increase in pro-inflammatory cytokines in the brain. As a result, these excessive pro-inflammatory cytokines cause the alterations in the structure and function of the CNS [16,17]. The circulating cytokines are considered as the important mediators in the gut to brain communication [18].

Increasing evidence supports the idea that the disturbance of gut-brain axis is deeply involved in the occurrence and progress of neurodevelopmental disorders [19,20]. Moreover, the pro-inflammatory cytokines have been recognized as key messengers of the communication between the gut and the brain [21]. The intestinal barrier and the BBB, including the blood-cerebrospinal fluid barrier (BCSFB), have significant roles in the gut-brain axis [22,23]. The intestinal barrier restricts the penetration of various noxious substances [24], and the BBB and the BCSFB regulate the passage between the blood and the brain to keep harmful substances from entering the brain [25]. The abundant tight junction proteins (TJs), such as zonula occludens-1 (ZO-1) and claudin-1 (CLDN-1), are required to maintain the permeability of intestinal barrier, as well as BBB [26,27].

In the present study, rats were subjected to NP exposure in their early lives by maternal gavage. The prefrontal cortex, a key region of the brain, is associated with the cognitive function, memory forming, emotion control, language comprehension, and attention [28–30]. Impairment of this region is believed to be involved in varied neurodevelopmental disorders [28]. We explored the effects of maternal NP exposure on interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) in the prefrontal cortices of rats. In addition, we also investigated these pro-inflammatory cytokines in their intestines, as well as in serums. The permeability of intestinal barrier, BBB, and BCSFB, critical barriers that are crucial for the gut to brain communication of pro-inflammatory cytokines, was examined. Finally, we determined the alteration in the expression of ZO-1 and CLDN-1, two important TJs that account for maintaining the permeability of intestinal barrier and BBB. Rats exposed to valproic acid (VPA) in early lives are widely used as animal models of neurodevelopmental disorders [31]. Moreover, exposure to VPA in early life increases the levels of the pro-inflammatory cytokines in the brain and the intestine [32,33], and it also leads to impairments of the intestinal barrier and BBB [33,34]. Thus, VPA-exposed rats were observed as positive controls in the present study.

2. Materials and methods

2.1. Animals

Sprague Dawley rats (230–250 g) were obtained from the Center for Experimental Animals at China Medical University (Shenyang, China) with a National Animal Use License number of SYXK-LN2013-0007. Animal use and all experiments and procedures involving animals were approved by the Animal Care and Use Committee at China Medical University, which complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and reduce the number of animals used. Rats were housed at a temperature of 24 ± 1 °C with 12 h light/12 h dark cycles and humidity at 50–60%. Food and water were provided *ad libitum*. Animals were housed for 1 week before being entered into the study. The female rats were randomly assigned into four groups (20 dams in per group), and placed in individual cages alone. Subsequently, they were mated with male rats (♀/♂ = 1:1). The day of appearance of the vaginal plug was regarded as gestation day 0 (GD 0). On GD12, the pregnant rats in the VPA treatment group were intraperitoneally injected with 600 mg/kg VPA (Sigma, USA). From GD 0 till postnatal day (PND) 21, the pregnant rats in other groups were subjected to NP (purity > 99%, Acros Organics, Morris, USA) (dissolved in corn oil) daily by oral gavage at 0, 10, or 50 mg/kg. All the dams were allowed to feed their pups until weaning on PND 21. To avoid any bias due to by sex or estrogen, only male pups were included in the follow-up study. No observed adverse effect level (NOAEL) of NP has been reported as 50 mg/kg/day in the rat model [35,36]. Hence, we treated the dams with NP at the doses of 0, 10, and 50 mg/kg/day respectively.

2.2. Enzyme-linked immunosorbent assay (ELISA)

ELISA was conducted according to the manufacturer's instructions. In brief, the pups were anesthetized with the intraperitoneal injection of sodium pentobarbital on PND42. The intestine and the prefrontal cortex tissues were dissected from the rats respectively. All the tissues were cut into pieces and homogenized. The homogenate was centrifuged (10,000 rpm) at 4 °C for 20 min, and the supernatant was collected. The protein concentration was measured using the BCA protein as-

say kit (Beyotime, China). The serum was separated from whole blood by centrifuged (3000 rpm) at 4 °C for 10 min and stored at −80 °C. The levels of IL-1β, IL-6, and TNF-α in supernatant and serum were quantified by ELISA kits (Meimian, China). The absorbance at 450 nm was measured by a multifunctional microplate reader (H1MD, BioTek, USA).

2.3. Determination of BBB and BCSFB permeability

BBB permeability was detected according to the method as described previously by N. Gorle. et al. [23]. All the following operations were kept away from light. Briefly, 4-kDa fluorescein isothiocyanate-dextran (FITC-dextran) (Sigma, USA) was diluted in 0.9% physiological saline. The 4-kDa FITC-dextran was injected into the caudal vein of pups (50 mg/kg) on PND42. After 40 min, pups were anesthetized with the intraperitoneal injection of sodium pentobarbital. Then, they were perfused with 0.9% physiological saline, and the brains were removed from the skull. The prefrontal cortex was obtained from the brain on ice rapidly. Next, the tissue was grounded in 50% trichloroacetic acid buffer solution on ice and then centrifuged (10,000 rpm) at 4 °C for 20 min to obtain the supernatant. The fluorescence intensity was detected by using the multifunctional microplate reader set to wavelength of λex/λem = 488/528 nm. The fold changes of the fluorescence intensity compared to that in the control group were calculated to demonstrate the alteration in BBB permeability.

BCSFB permeability was detected according to the method as described previously by M. Brkic. et al. [37]. Other pups were also treated as mentioned above, and their cerebrospinal fluids were obtained from the fourth ventricle using the cisterna magna puncture method. The BCSFB permeability was determined as mentioned above.

2.4. Determination of intestinal barrier permeability

The intestinal barrier permeability was detected according to the method as described previously by F. Van Hauwermeiren. et al. [38]. The pups were fasted for 24 h before the permeability assay. All the following operations were kept away from light. In brief, 4-kDa FITC-dextran was diluted in 0.9% physiological saline. The pups were subjected to 4-kDa FITC-dextran by intragastric administration (150 mg/kg). After 5 h, they were anesthetized with the intraperitoneal injection of sodium pentobarbital. The pulsatile heart blood was collected and centrifuged (3000 rpm) at 4 °C for 10 min to obtain the serum. The fluorescence intensity was analyzed by using the multifunctional microplate reader set to wavelength of λex/λem = 488/528 nm. The fold changes of the fluorescence intensity compared to that in the control group were calculated to demonstrate the alteration in intestinal barrier permeability.

2.5. Western blotting

The pups were anesthetized with the intraperitoneal injection of sodium pentobarbital. The prefrontal cortex tissues were isolated and washed in ice-cold artificial cerebrospinal fluid on PND42. The intestine tissues were isolated and washed in ice-cold 0.9% physiological saline. These tissues were homogenized with the ultrasonic tissue disrupter in cold RIPA lysis buffer containing phosphatase inhibitors and protease. The supernatants of samples were collected after centrifuging (10,000 rpm) at 4 °C for 20 min. The protein concentration was determined using the BCA protein assay kit (Beyotime, China) and adjusted to the same concentration (3 μg/μl). Protein samples were loaded on a 10% sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). At the end of the electrophoresis, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The PVDF membranes were blocked in 5% nonfat milk (diluted in Tris-buffered saline with Tween 20) for 1.5 h. Afterward, the membranes were incubated with primary antibodies of ZO-1 (Proteintech; 1:500, USA) and CLDN-1(Santa Cruz; 1:500, USA) at 4 °C overnight. Next, the membranes were incubated with the secondary antibodies (Zhongshan Biotechnology; goat anti-rabbit, 1:3000, China) or (Zhongshan Biotechnology; goat anti-mouse, 1:3000, China) at room temperature for 1 h. Intensities of immunoreactive bands were detected by Bioanalytical Imaging System (Azure Biosystems, USA) and quantified by Image J software (NIH, USA). The target protein/β-actin ratios were calculated for comparison.

2.6. Immunofluorescence

The pups were anesthetized with the intraperitoneal injection of sodium pentobarbital and perfused with 0.9% physiological saline on PND42. Each rat was immediately euthanized, and the prefrontal cortex and the intestinal segment were obtained. The contents in the intestine were dislodged clearly and carefully, and the damages to the intestinal segments were avoided. All the tissues were submerged into 4% paraformaldehyde overnight. The fixed brains and intestinal tissues were embedded in paraffin, and serially sectioned into sections (4 μm thickness) by a rotary microtome (Leica, Germany). After deparaffinization and hydration in xylene and serial ethanol, slices were washed in phosphate-buffered saline (PBS) 3 times. After preincubation in 10% goat serum at room temperature for 30 min, the samples were incubated in primary antibodies of ZO-1 (Proteintech; 1:50, USA) and CLDN-1 (Santa Cruz; 1:50, USA) at 4 °C respectively. The slices were washed 3 times and incubated with fluorescently labeled 594-conjugated secondary antibody (ThermoFisher, 1:200, USA), or fluorescently labeled 488-conjugated secondary antibody (ThermoFisher, 1:200, USA) at room temperature for 2 h. The nuclei were stained by DAPI (Beyotime, China). Finally, the slices were observed using a fluorescence microscope (Olympus/IPP, Japan). The pictures were obtained under the same conditions at a magnification of 200 × (objective 20 × and ocular 10 ×).

2.7. Statistical analysis

All analyses were carried out using SPSS software (version 21.0, SPSS, USA). All tests were performed in at least triplicate. The results were expressed as mean ± standard error of the mean (SEM). Statistically significant values were assessed by one-way analysis of variance and Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Maternal exposure to NP increased the levels of pro-inflammatory cytokines in the prefrontal cortex of pups

The elevated levels of pro-inflammatory cytokines are linked to the neurodevelopmental disorders [39,40]. We analyzed the levels of IL-1β, IL-6, and TNF-α in the prefrontal cortex and serum by ELISA assays. For pro-inflammatory cy-

tokines in the prefrontal cortex, we found that the levels of IL-1β were significantly increased in 10 mg/kg NP and VPA groups compared to the control group ($P < 0.05$, Fig. 1A). There was no significant difference in the levels of IL-1β between the 50 mg/kg NP group and the control group. The IL-6 levels were significantly increased in the 10, 50 mg/kg NP, and VPA groups compared to the control group ($P < 0.05$, Fig. 1B). The TNF-α level was significantly increased in the VPA group compared to the control group ($P < 0.05$, Fig. 1C). The TNF-α levels in 10 and 50 mg/kg NP groups tended to be higher than the control group, but the differences were not significant.

3.2. Maternal exposure to NP increased the levels of pro-inflammatory cytokines in the serum of pups

Maternal exposure to 10, 50 mg/kg NP, and VPA significantly increased the IL-1β levels in the serum compared to the control group ($P < 0.05$, Fig. 1D). Serum IL-6 levels were significantly increased in the 10 mg/kg NP and VPA groups compared to the control group ($P < 0.05$, Fig. 1E). Serum IL-6 level in the 50 mg/kg NP group was also higher than the control group, although the difference was not significant. Serum TNF-α level was significantly increased in the VPA group compared to the control group ($P < 0.05$, Fig. 1F). However, maternal exposure to 10 and 50 mg/kg NP did not increase the serum TNF-α level compared to the control group.

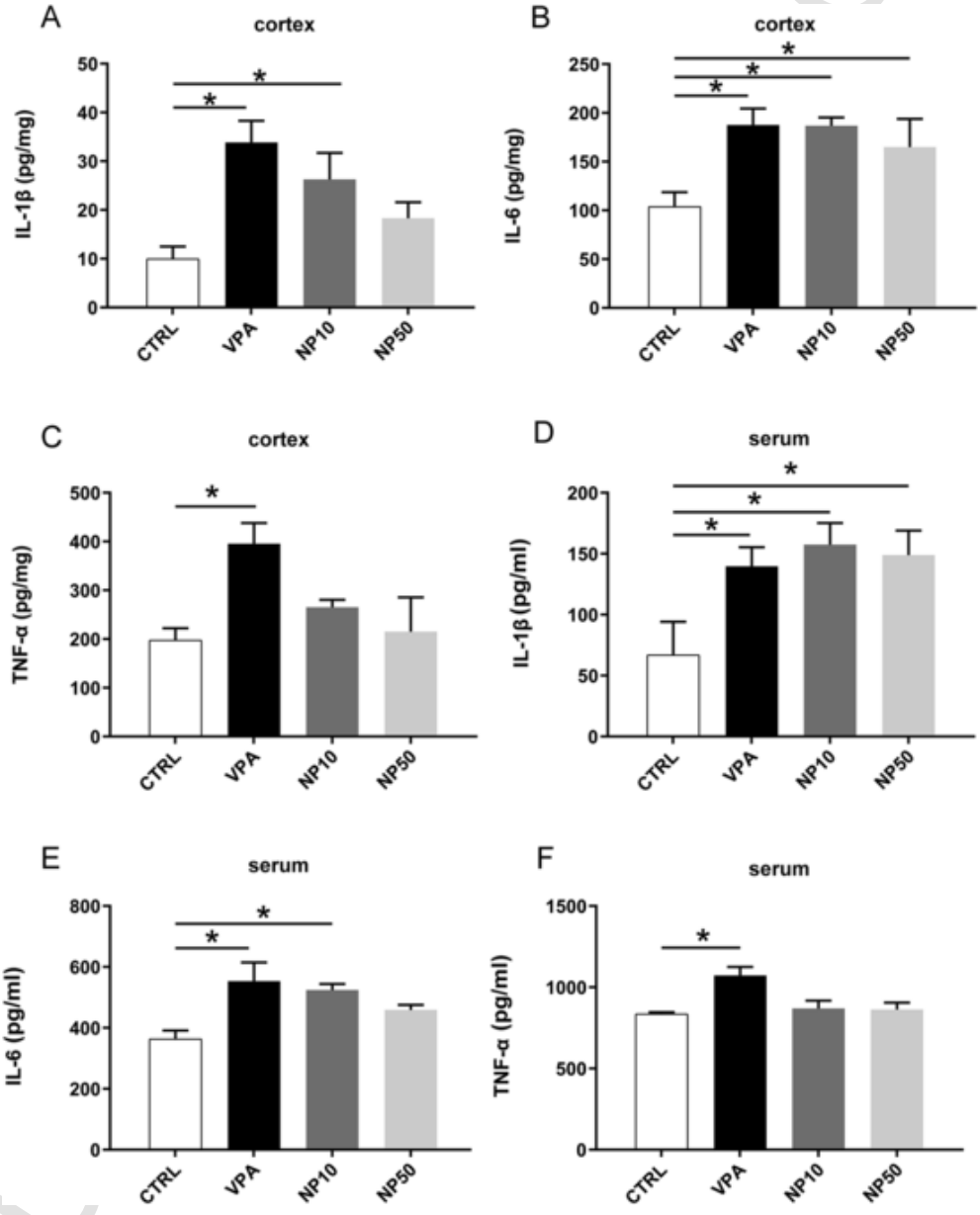

Fig. 1. Maternal exposure to NP increased the levels of pro-inflammatory cytokines in the prefrontal cortex and serum of pups. The levels of IL-1β (A), IL-6 (B), and TNF-α (C) in the prefrontal cortex. The levels of IL-1β (D), IL-6 (E), and TNF-α (F) in the serum. Each bar represents mean ± SEM (n = 5–6). *$P < 0.05$ compared to the control.

3.3. Maternal exposure to NP increased the BBB and BCSFB permeability of pups

The BBB and BCSFB permeability is in charge of regulating the passage of solutes and fluids between the circulatory system and the brain [41]. Emerging evidence supports the idea that pro-inflammatory cytokines that cross the BBB and BCSFB may lead to neuroinflammation [42]. The BBB permeability in the 10 mg/kg NP and VPA groups was significantly increased compared to the control group ($P < 0.05$, Fig. 2A). Furthermore, the BBB permeability in the 50 mg/kg NP group was also increased compared to the control group, although the difference was not significant. The BCSFB permeability was significantly increased in the VPA group compared to the control group ($P < 0.05$, Fig. 2B). Maternal exposure to 10 and 50 mg/kg NP tended to increase the BCSFB permeability of pups.

3.4. Maternal exposure to NP decreased the expression of ZO-1 and CLDN-1 in the prefrontal cortex of pups

ZO-1 and CLDN-1, two well-known TJs, play critical roles in maintaining the permeability of BBB [43]. We evaluated the protein expression of ZO-1 and CLDN-1 in the prefrontal cortex of pups by Western blot. The expression of ZO-1 and CLDN-1 was significantly decreased in the prefrontal cortex of pups subjected to 10 mg/kg NP and VPA treatment compared to the controls ($P < 0.05$, Fig. 3A–D). Meanwhile, the level of CLDN-1 was significantly decreased in the 50 mg/kg NP group compared to the control group ($P < 0.05$). We also observed the expression of these two TJs in the prefrontal cortex by immunofluorescence staining, which showed similar results to Western blot (Fig. 3E and F).

3.5. Maternal exposure to NP induced the production of pro-inflammatory cytokine in the intestine of pups and increased the intestinal barrier permeability

The immune cells in the intestine during chronic inflammation produce various kinds of pro-inflammatory substances, such as cytokines, which subsequently enter the circulation via the intestinal barrier [42]. Our results indicated that the levels of IL-1β, IL-6, and TNF-α in the intestines were significantly increased in the 10, 50 mg/kg NP, and VPA groups compared to the control group ($P < 0.05$, Fig. 4A–C). Moreover, the intestinal barrier permeability was significantly increased in 10 mg/kg NP and VPA groups compared to the control group ($P < 0.05$, Fig. 4D). The intestinal barrier permeability of the 50 mg/kg NP group was slightly higher than the control group.

3.6. Maternal exposure to NP decreased the expression of ZO-1 and CLDN-1 in intestine of pups

The TJs ZO-1 and CLDN-1 are deeply involved in maintaining the permeability of intestinal barrier, in addition to the permeability of BBB [44]. The expression of ZO-1 and CLDN-1 in the intestines of pups was analyzed by Western blot, and the results indicated that the expression of ZO-1 and CLDN-1 was significantly decreased in 10, 50 mg/kg NP, and VPA groups compared to the control group ($P < 0.05$, Fig. 5A–D). We also investigated expression of these two TJs in intestines by immunofluorescence staining, which showed similar results to Western blot (Fig. 5E and F).

4. Discussion

There are growing concerns about the neurodevelopmental impairments caused by exposure to POPs in early life [45,46]. As a typical POP, NP exposure during the critical period of the CNS development has been found to negatively affect the development of many brain regions, such as hippocampus, cerebellum, and cortex [47,48]. Furthermore, it has been reported that perinatal exposure to NP impaired various functions of the CNS, such as learning and memory, which were largely irreversible in adolescence or adulthood [49]. Notably, the abnormal increase in the levels of pro-inflammatory cytokines has been linked to the impairments of neurodevelopment and the CNS functions. In the present study, the animal model of exposure to NP in early life was established by maternal gavage during the pregnancy and lactation. We investigated the levels of IL-1β, IL-6, and TNF-α in the prefrontal cortex, as well as serum and intestine. We also investigated the permeability of intestinal barrier and BBB, which are two crucial barriers in the gut to brain communication.

Neuroinflammation is well known to involve in neurodevelopmental disorders, such as schizophrenia, autism spectrum disorder (ASD), and attention deficit and hyperactivity disorder (ADHD) [50–52]. Moreover, excessive pro-inflammatory cytokines in the brain, particularly in early life, exert detrimental effects on the CNS structure and function [53]. The increased concentrations of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, have been observed in brain of individuals and animals that suffer from neurodevelopmental dis-

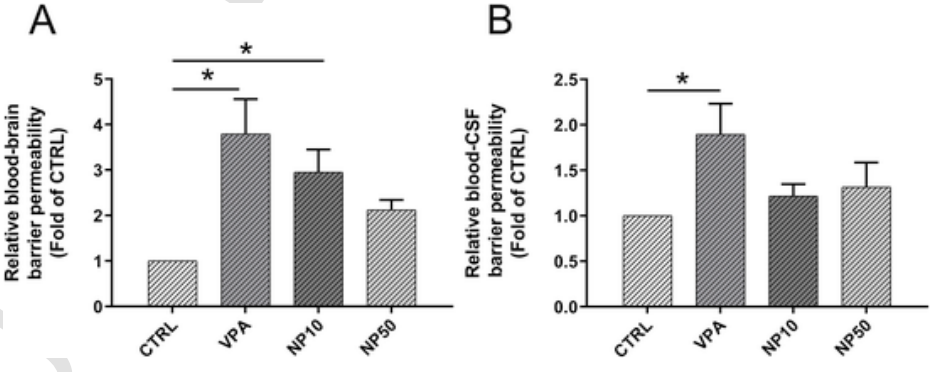

Fig. 2. Maternal exposure to NP increased the BBB and BCSFB permeability of pups. Maternal exposure to NP increased the BBB permeability (A). Maternal exposure to NP tended to increase the BCSFB permeability (B). Each bar represents mean ± SEM (n = 6). *$P < 0.05$ compared to the control.

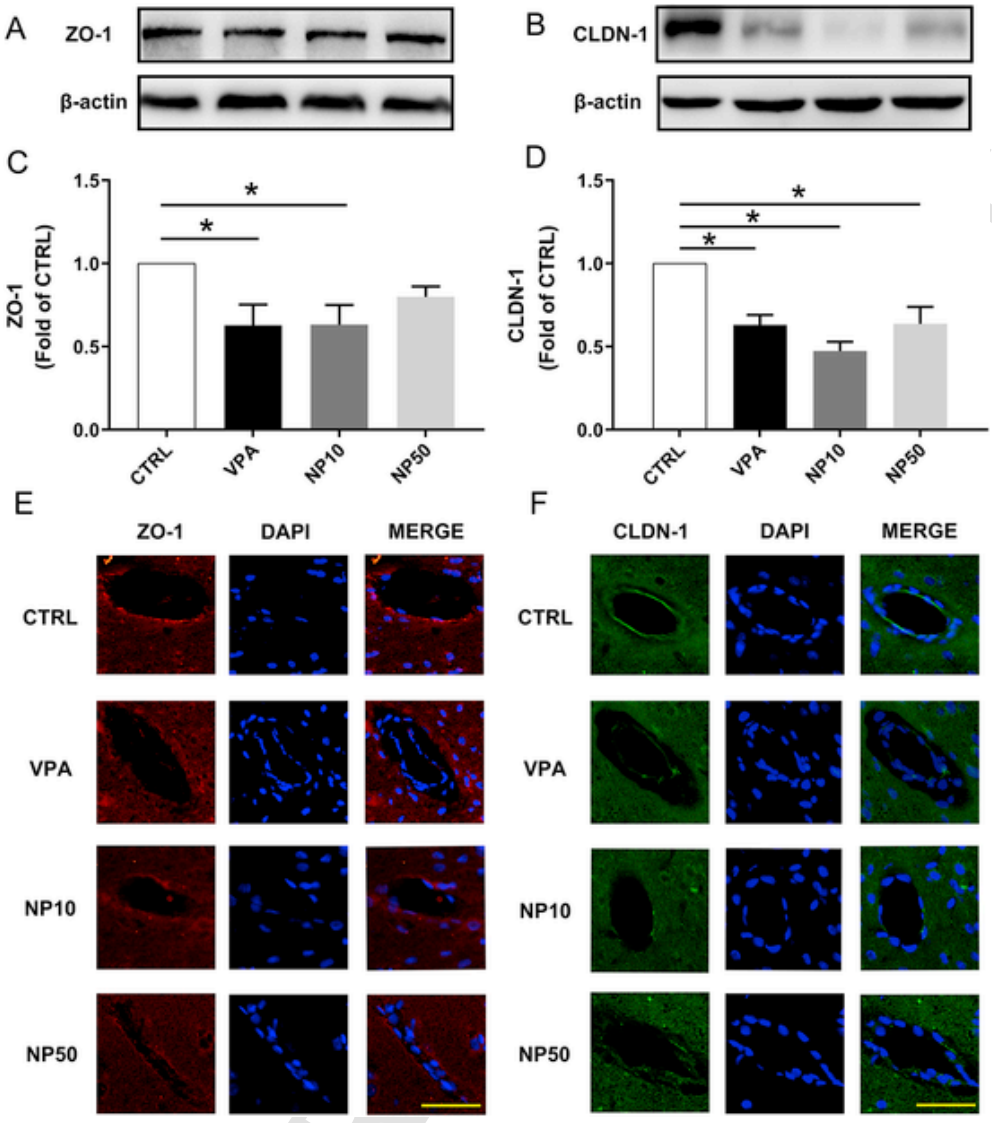

Fig. 3. Maternal exposure to NP decreased the expression of ZO-1 and CLDN-1 in the prefrontal cortex of pups. Representative images of Western blot using antibodies against ZO-1 (A) and CLDN-1 (B) in the prefrontal cortex of pups. The bar graphs showed the semi-quantitative measurement of ZO-1 (C) and CLDN-1 (D) in the prefrontal cortex as fold changes compared with the controls. Representative images showed the fluorescent staining of ZO-1 (E) in the prefrontal cortex of pups. The sections of prefrontal cortex were stained with rabbit anti-ZO-1 (red) and DAPI (blue). Representative images showed the fluorescent staining of CLDN-1 (F) in the prefrontal cortex of pups. The sections of prefrontal cortex were stained with mouse anti-CLDN-1 (green) and DAPI (blue). Scale bar = 50 μm. Each bar represents mean ± SEM (n = 6). *$P < 0.05$ compared to the control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ability [54,55]. The cortex is a key brain region that has been studied extensively in the occurrence and progress of neurodevelopmental disorders [56]. Excessive pro-inflammatory cytokines have been found in the cortical regions of individuals or animals with neurodevelopmental disability [57,58]. Maternal exposure to NP has been shown to increase the levels of IL-1β, IL-6, and TNF-α in offspring hippocampus of rats [59]. In the present study, exposure to NP in early life also increased the levels of IL-1β and IL-6 in the prefrontal cortex, while the level of TNF-α did not markedly change. Similarly, the maternally VPA-exposed pups showed the elevation of pro-inflammatory cytokine levels in the prefrontal cortex. This result is consistent with the previous study, which indicated that exposure to VPA in early life of rat (as an excellent tool to establish animal model of neurodevelopmental disability) induced the increase in pro-inflammatory cytokines levels in the brain [32].

The increased levels of circulating pro-inflammatory cytokines may directly contribute to the elevation of levels in the brain, especially when the brain barriers collapse [60]. For example, circulating pro-inflammatory cytokines of patients with schizophrenia are shown to enter the brain due to the leakage of the BBB [61]. Interestingly, we found that the pro-inflammatory cytokines levels were increased in the serums of pups that were exposed to NP or VPA in early lives. This is in line with the previous observation of the increased levels of pro-inflammatory cytokines in the serums of mice exposed to NP [62]. It should be noted that the alteration of pro-inflammatory cytokines levels in the serum and in the brain displayed quite a similar trend in the present study. The BBB is a critically physical and biochemical barrier that is responsible

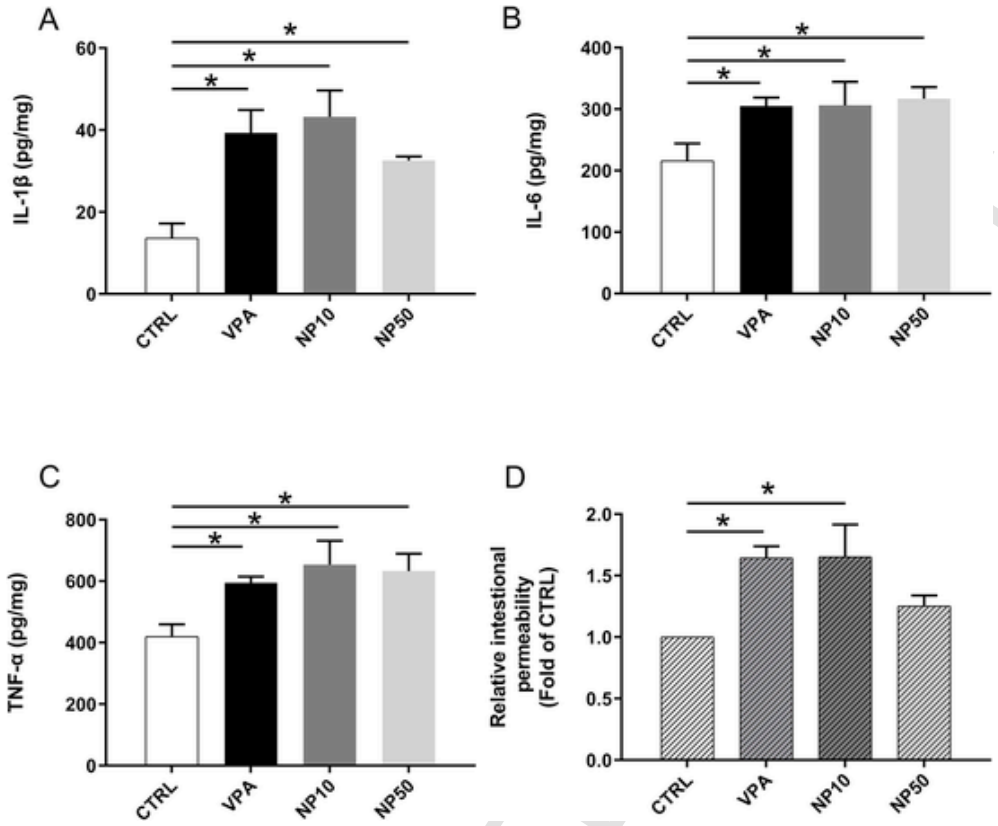

Fig. 4. Maternal exposure to NP induced pro-inflammatory cytokine production in the intestine of pups and increased the intestinal barrier permeability. The levels of IL-1β (A), IL-6 (B), and TNF-α (C) in the offspring intestines. Maternal exposure to NP increased the permeability of intestinal barrier (D). Each bar represents mean ± SEM (n = 5–6). *$P < 0.05$ compared to the control.

for maintaining the CNS homeostasis [63]. Our results showed that perinatal exposure to NP or VPA enhanced BBB permeability in the offspring brain. Taken together, these data suggested that the increased levels of pro-inflammatory cytokines in the prefrontal cortex caused by exposure to NP or VPA in early life may be due to the damage of BBB permeability, and the increase in these cytokines in the serum.

The increased levels of pro-inflammatory cytokines in the serum may derive from the elevation of pro-inflammatory cytokines in the intestine, especially when the intestinal barrier permeability is damaged [64]. Excessive intestinal pro-inflammatory cytokines could enter the circulatory system and influence their levels in distant sites, such as the CNS [65]. Moreover, the alteration of intestinal barrier permeability has been linked to some neurodevelopmental disorders [66]. Our results showed that the IL-1β, IL-6, and TNF-α levels in the intestine were significantly increased by NP or VPA treatment in early life. Furthermore, these treatments also increased the permeability of intestinal barrier. These results suggested that the increased levels of pro-inflammatory cytokines in the intestine and the damages of intestinal barrier permeability may contribute to the elevation of these cytokines in the serum caused by exposure to NP or VPA in early life.

ZO-1 is a scaffolding protein, which locates on the cytoplasmic surface of tightly connected endothelial cells [67]. CLDN-1 is an integral membrane TJ between epithelial cells, which regulates paracellular transport and maintains cell polarity [68]. Both ZO-1 and CLDN-1, as typical TJs, are required to maintain the integrity and function of BBB and intestinal barrier. The loss of ZO-1 and CLDN-1 has been shown to increase the permeability of the BBB and the intestinal barrier [69–72]. In the present study, exposure to NP or VPA decreased ZO-1 and CLDN-1 expression in the prefrontal cortex, as well as in the intestine. These results supported the idea that the reduction of the ZO-1 and CLDN-1 were involved in the increased permeability of BBB and intestinal barrier.

It is widely accepted that the disturbance of the gut-brain axis is associated with the neurodevelopmental disorders [73]. Even if the direct relationship between the intestine and the brain are difficult to imagine, these two systems are intimately connected [74]. The immune system could act as an important bridge, while cytokines could serve as messengers or mediators in this close relationship [75]. In the present study, exposure to NP or VPA in early life increased the levels of pro-inflammatory cytokines in the prefrontal cortex, as well as in the serum and the intestine. The permeability of intestinal barrier and BBB, two critical barriers in the gut to brain communication, was also increased. The decreased expression of ZO-1 and CLDN-1, TJs that account for maintaining the permeability of intestinal barrier and BBB, may underlie these increases in permeability. These lines of evidence suggested that the disturbance of gut-brain communication may contribute to the increase in pro-inflammatory cytokines in the prefrontal cortex induced by exposure to NP in early life.

CRediT authorship contribution statement

Xiaoyu Che: Formal analysis, Investigation, Methodology, Writing - original draft. **Yawen Fang:** Formal analysis, Investigation, Methodology. **Mingdan You:** Methodology, Validation.

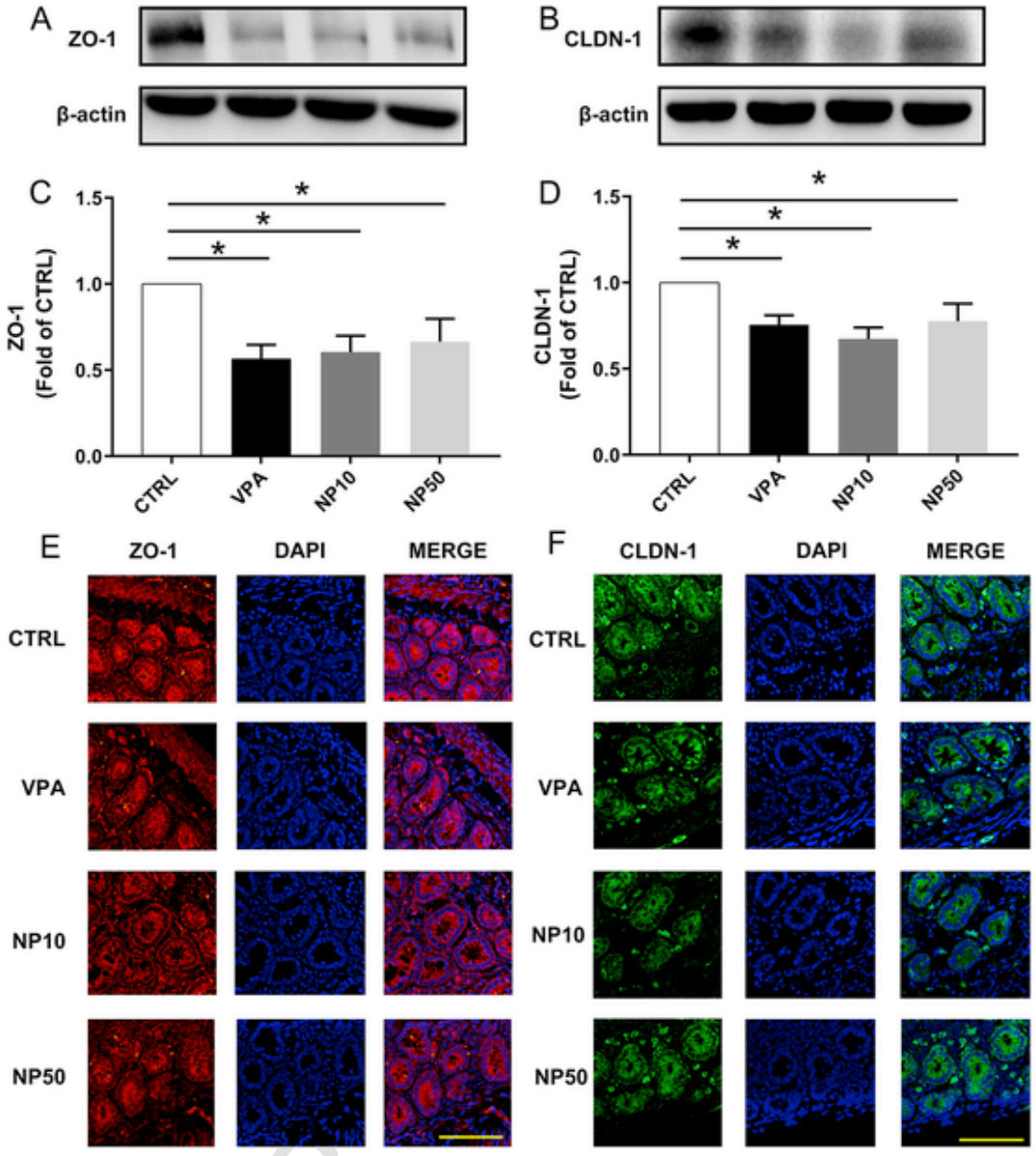

Fig. 5. Maternal exposure to NP decreased the expression of ZO-1 and CLDN-1 in the intestine of pups. Representative images of Western blot using antibodies against ZO-1 (A) and CLDN-1 (B) in intestines of pups. The bar graphs showed the semi-quantitative measurement of ZO-1 (C) and CLDN-1 (D) in intestines as fold changes compared with controls. The representative images showed the fluorescent staining of ZO-1 (E) in the intestines of pups. The sections of intestines were stained with rabbit anti-ZO-1 (red) and DAPI (blue). Representative images showed the fluorescent staining of CLDN-1 (F) in the intestines of pups. The sections of intestines were stained with mouse anti-CLDN-1 (green) and DAPI (blue). Scale bar = 100 μm. Each bar represents mean ± SEM (n = 6). *$P < 0.05$ compared to the control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Yuanyuan Xu: Data curation, Writing - review & editing. **Yi Wang:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of competing 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 papers.

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