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Prenatal exposure to valproic acid causes allodynia associated with spinal microglial activation

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ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in social communication and social interaction and the presence of restricted, repetitive behaviors. Additionally, difficulties in sensory processing commonly occur in ASD. Sensory abnormalities include heightened or reduced sensitivity to pain, but the mechanism underlying sensory phenotypes in ASD remain unknown. Emerging evidence suggests that microglia play an important role in forming and refining neuronal circuitry, and thus contribute to neuronal plasticity and nociceptive signaling. In the present study, we investigated the age-dependent tactile sensitivity in an animal model of ASD induced by prenatal exposure to valproic acid (VPA) and subsequently assessed the involvement of microglia in the spinal cord in pain processing. Pregnant ICR (CD1) mice were intraperitoneally injected with either saline or VPA (500 mg/kg) on embryonic day 12.5. Male offspring of VPA-treated mothers showed mechanical allodynia at both 4 and 8 weeks of age. In the spinal cord dorsal horn in prenatally VPA-treated mice, the numbers and staining intensities of ionized calcium-binding adapter molecule 1-positive cells were increased and the cell bodies became enlarged, indicating microglial activation. Treatment with PLX3397, a colony-stimulating factor 1 receptor inhibitor, for 10 days resulted in a decreased number of spinal microglia and attenuated mechanical allodynia in adult mice prenatally exposed to VPA. Additionally, intrathecal injection of Mac-1-saporin, a saporin-conjugated anti-CD11b antibody to deplete microglia, abolished

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mechanical allodynia. These findings suggest that prenatal VPA treatment causes allodynia and that spinal microglia contribute to the increased nociceptive responses.

Abbreviations

ASD	autism spectrum disorder
VPA	valproic acid
PBS	phosphate-buffered saline
ANOVA	analysis of variance
DAPI	4′,6-diamidino-2-phenylindole
Iba1	Ionized calcium-binding adapter molecule 1
CSF1R	colony-stimulating factor 1 receptor
HDAC	histone deacetylase

1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by core symptoms that include impairments in social behavior and communication, as well as restricted and repetitive behaviors (Geschwind and Levitt, 2007; Persico and Bourgeron, 2006). Additionally, individuals with ASD exhibit aberrant reactivity to sensory stimuli such as mechanical tactile stimuli. Notably, pressure pain thresholds were lower in autistic individuals compared to controls (Fan et al., 2014). About 61% of patients with ASD report altered tactile sensitivity in both glabrous (smooth) and hairy skin (Tomchek and Dunn, 2007) and increased sensitivity to vibration and thermal pain (Cascio et al., 2008; Blakemore et al., 2006). However, clinical findings about behavioral responses to noxious and non-noxious stimuli might be inconsistent and hyposensitivity, hypersensitivity, or both can be exhibited in children with ASD (Allely, 2013; Failla et al., 2020; Schaffler et al., 2019; Riquelme et al., 2016; Yasuda et al., 2016; Baranek et al., 2006). Hypersensitivity of peripheral neurons may contribute to the avoidance of social touch, a common behavioral phenotype in individuals with ASD (Pellecchia et al., 2016; Voos et al., 2013), whereas hyposensitivity in the peripheral nervous system may result in an inadequate amount of touch information reaching the brain, causing individuals to be indifferent to social touch (Schaffler et al., 2019). Therefore, elucidating the causes and effects of somatosensory differences is important for understanding the etiology of ASD and associated symptoms.

Several environmental risk factors such as prenatal exposure to agents play a critical role in the altered brain development leading to ASD. Maternal use of valproic acid (VPA) during pregnancy has been implicated in the etiology of ASD in children (Christensen et al., 2013; Ornoy, 2009). Rodents prenatally exposed to VPA show behavioral alterations, such as deficits in social interaction, similar to those observed in humans with ASD (Hara et al., 2016, 2017; Yamaguchi et al., 2017; Kataoka et al., 2013; Wagner et al., 2006; Schneider and Przewłocki, 2005; Juybari et al., 2020), and thus have been used as an animal model of ASD. Additionally, a previous study showed that prenatal exposure to VPA caused mechanical allodynia in male rats (Schneider et al., 2006). However, the mechanisms underlying the altered tactile sensitivity in prenatally VPA-exposed animals remain unclear.

Accumulating evidence suggests that activated microglia in the spinal cord play a critical role in mechanical allodynia following peripheral nerve injury (Marchand et al., 2005; Tsuda et al., 2005; Tsuda, 2019; Watkins et al., 2001; Inoue and Tsuda, 2021; Nakamura et al., 2021). Microglia also play a crucial role in forming and refining neuronal circuitry, and thus contribute to neuronal plasticity and nociceptive signaling (Andoh and Koyama, 2021; Wilton et al., 2019). Recently, prenatally VPA-treated mice were shown to exhibit an increased number and/or volume of microglia in the prefrontal cortex, hippocampus, and lateral septum (Traetta et al., 2021; de Leão et al., 2021; Dos Santos et al., 2021). It has also been reported that prenatal exposure of mice to VPA affects microglial cell density in the brain in a region-specific manner during postnatal development (Gifford et al., 2022). However, it remains to be determined whether microglia in the spinal cord are altered in prenatally VPA-exposed animals and, more importantly, whether spinal microglia contribute to prenatal VPA-induced mechanical allodynia. In the present study, we investigated the age-dependent tactile sensitivity in a prenatal VPA-induced model of ASD and subsequently assessed the involvement of microglia in pain processing.

2. Materials and methods

2.1. Animals and drug administration

Experimental procedures that involved animals and their care were conducted in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and ARRIVE guidelines (McGrath and Lilley, 2015; Kilkenny et al., 2010). All animal experiments were approved by the Committee of Research Facilities for Laboratory Animal Science of Hiroshima University (#A20-115). Female ICR (CD1) mice (Japan SLC, Inc., Shizuoka, Japan) were obtained at 6 days of gestation and housed individually in plastic cages under a standard 12-h light/dark cycle (lights on 08:00 h) at a constant temperature of 22 ± 1 °C. The animals had *ad libitum* access to food (standard rodent chow, MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water.

The pregnant mice were intraperitoneally injected with 500 mg/kg of either VPA (Sigma-Aldrich, St. Louis, MO, USA) or saline (0.9% NaCl solution; Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) on embryonic day 12.5 (E12.5) (Kataoka et al., 2013; Kawase et al., 2019; Hara et al., 2016, 2017; Yamaguchi et al., 2017). VPA was dissolved in saline and the injected volume was 10 ml/kg. All animals were returned to their home cages immediately after the injection and left undisturbed until weaning of the offspring. Offspring born to VPA- and saline-treated mothers were weaned, sexed, and caged in groups of five mice of the same sex at postnatal day 21. In this study, we used only male offspring to avoid sex differences that we reported in previous publications (Hara et al., 2012, 2015; Kataoka et al., 2013).

2.2. Von Frey test

Withdrawal thresholds to mechanical stimuli were measured using a von Frey test at the ages of 4 and 8 weeks. The von Frey test was performed under constant conditions (23 ± 2 °C; 55 ± 5% humidity) between 09:00 and 13:00 h in a quiet room. Mechanical allodynia was assessed by the ascending stimulus method (Deuis et al., 2017). Mice were acclimated to the experimental environment by placing them on an elevated wire grid (5 × 5 mm) for 1 h prior to the start of testing. Pressure was applied to the hind paw plantar surface using von Frey filaments (Tactile Test Aesthesio Semmes-Weinstein Von Frey Aesthesiometer; Muromachi Kikai Co., Ltd., Tokyo, Japan) of different thicknesses (0.02, 0.04, 0.07, 0.16, 0.40, 0.60, 1.00, 1.40, and 2.00 g strength: starting with the 0.02 g filament). In the paradigm of the ascending stimulus method, the test begins by assessing the response to a filament with a force of 0.02 g applied five times. Quick withdrawal, shaking, biting, and licking of the stimulated paw were regarded as positive paw withdrawal responses (Saika et al., 2020). If the response rate was less than 40%, the next filament was tested. If the response rate was 40% or more, testing stopped and the force of the last von Frey

filament was designated as the mechanical withdrawal threshold. All experiments were conducted by experimenters who were blind to the treatment conditions of the animals.

2.3. Depletion of microglia

The colony-stimulating factor 1 receptor (CSF1R) is critical for microglia development; microglia are almost completely absent in *Csf1r*-deficient mice (Erblich et al., 2011; Ginhoux et al., 2010). Moreover, maintenance of adult microglia requires CSF1R signaling; pharmacological inhibition of CSF1R in the adult largely eliminates microglia (Elmore et al., 2014). Therefore, we used a CSF1R inhibitor PLX3397 to induce global depletion of microglia. A robust and time-dependent reduction in brain microglia number, with a 50% reduction in microglia, was observed after just 3 days of treatment with PLX3397 (290 mg/kg chow) (Elmore et al., 2014). Additionally, the number of microglia in the brain was stably reduced by more than 70% over 1–2 weeks of the PLX3397 treatment. Thus, in this study, mice were treated with PLX3397 for 10 days. PLX3397 was incorporated into the AIN-76A rodent diet (Research Diets, Inc., New Brunswick, NJ, USA) at 290 mg/kg chow and administered to adult (8-week-old) mice for 10 days. The dose of PLX3397 and its route of administration were set in accordance with previous studies (Saika et al., 2021; Elmore et al., 2014). The AIN-76A rodent diet was used as a control. To deplete microglia in the spinal cord, Mac-1-saporin (saporin-conjugated anti-CD11b antibody) (11.2 μg/5.5 μl), a microglia-selective toxin, was injected intrathecally at the level of L4–L5 in adult (8-week-old) mice (Liang et al., 2019). Vehicle groups were injected with the same volume of saline. The von Frey test was conducted 1 day after Mac-1-saporin treatment.

2.4. Immunohistochemistry

The mice were deeply anesthetized with isoflurane and transcardially perfused with ice-cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde phosphate buffer solution (#09154-85; Nacalai Tesque, Inc., Kyoto, Japan). Then, the lumbar spinal cord (L4–L5) was dissected, post-fixed with the same fixative, and cryoprotected with 30% sucrose-containing PBS at 4 °C. The tissue was embedded in Tissue-Tek O.C.T. compound 4583 (Catalog # 45833; Sakura Finetech, Tokyo, Japan). Thirty-micrometer-thick sections were cut using a cryostat (Leica CM3050 S; Leica Biosystems, Wetzlar, Germany). Sections were permeabilized with 0.2% Triton X-100 in PBS for 20 min and blocked by 5% goat or donkey serum in PBS containing 0.03% Triton X-100 for 1 h at room temperature. Then, they were incubated with a rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1) polyclonal antibody (1:500, RRID: AB_839504; WAKO Pure Chemical Industries, Osaka, Japan) and/or a rat anti-CD68 monoclonal antibody (1:100, RRID:AB_32421; Bio-Rad Laboratories, Hercules, CA, USA) at 4 °C overnight, followed by an Alexa Fluor 488-labeled anti-rabbit IgG (1:200, RRID: AB_143165; Thermo Fisher Scientific, Waltham, MA, USA), an Alexa Fluor 594-labeled anti-rabbit IgG (1:200, RRID: AB_141637; Thermo Fisher Scientific) and/or an Alexa Fluor 488-labeled anti-rat IgG (1:200, RRID: AB_2737355; Abcam Cambridge, UK) for 2 h at room temperature. For CD11b staining, sections were heated in antigen retrieval solution (Catalog #S1699, DAKO, Carpinteria, CA, USA) for 1 h at 90 °C, permeabilized with 0.2% Triton X-100 in PBS for 20 min and blocked by 5% goat serum in PBS containing 0.03% Triton X-100 for 1 h at room temperature. Then, they were incubated with a rabbit anti-CD11b polyclonal antibody (1:500, Catalog # HS-384 103, Synaptic Systems, Göttingen, Germany) at 4 °C overnight, followed by an Alexa Fluor 488-labeled anti-rabbit IgG (1:200) for 2 h at room temperature. Sections were washed in PBS, mounted on slides, and coverslipped with Prolong Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific). Fluorescence images were acquired with a fluorescence microscope (BZ-X800; Keyence, Elmwood Park, NJ, USA). Iba1-positive microglia in laminae I–IV and V–VI of the dorsal horn of the spinal cord were counted per section on both sides, and the counts were averaged for each animal (Supplementary Fig. S1). All immunohistochemical experiments and analyses were performed in a blinded manner by experimenters who were unaware of the treatment conditions. Morphologies of microglia were quantified in three dimensions using the Neurolucida tracing system (MBF Bioscience, Williston, VT, USA) with the experimenter blinded to the treatment. A 60 × lens was used to analyze microglia morphology. The soma size and total number and length of microglial cell processes were compared among treatments using a NeuroExplorer (MBF Bioscience).

2.5. Statistics

All data are expressed as the mean ± standard error of the mean (S.E.M.). Data were analyzed by Student's *t*-test for comparison between two groups or by two-way analysis of variance (ANOVA), followed by the Tukey–Kramer test. Statistical analyses were conducted using the software package StatView® 5.0 for Windows (SAS Institute, Cary, NC, USA). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Mechanical allodynia in prenatally VPA-treated mice

We first examined the mechanical nociceptive threshold of male offspring prenatally treated with VPA (500 mg/kg) using the von Frey test (Fig. 1). Prenatally VPA-treated mice showed a significant decrease in withdrawal threshold compared with prenatally saline-treated control mice at the ages of 4 weeks (Fig. 1A) and 8 weeks (Fig. 1B), indicating mechanical allodynia.

3.2. Increased expression and activation of microglia in the dorsal horn of the spinal cord in prenatally VPA-treated mice

We next examined the expression of microglia in the spinal cord in mice prenatally treated with VPA (Fig. 2). Fig. 2A and B shows representative images of Iba1-positive microglia in laminae I–IV and V–VI of the dorsal horn of the spinal cord, respectively, in adult (8-week-old) mice. The numbers of microglia in laminae I–IV (Fig. 2C) and V–VI (Fig. 2E) in prenatally VPA-treated mice were increased compared with those in control mice. Increases in average intensity and cell area per microglia in laminae I–IV (Fig. 2D) and V–VI (Fig. 2F) were also observed in prenatally VPA-treated mice. We further identified increased levels of CD11b immunoreactivity in laminae I–IV (Fig. 3A)

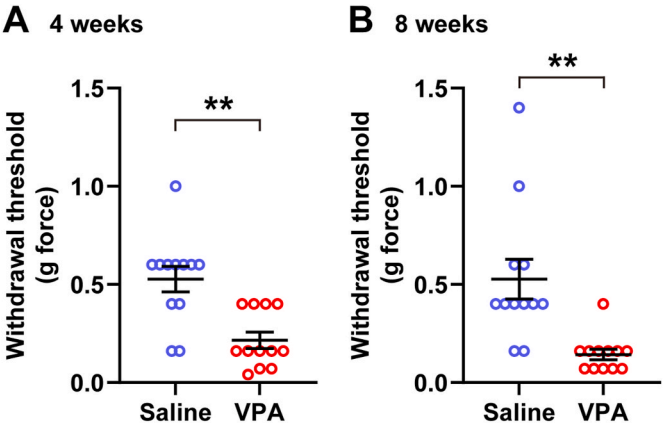

Fig. 1. Prenatal exposure to valproic acid (VPA) at embryonic day 12.5 (E12.5) causes mechanical allodynia in the von Frey test. Offspring born to mothers treated intraperitoneally with VPA (500 mg/kg) or saline at E12.5 were subjected to behavioral analysis at the ages of 4 (A) and 8 weeks (B). Results are expressed as the mean ± S.E.M. of 12 mice per group. $^{**}P < 0.01$.

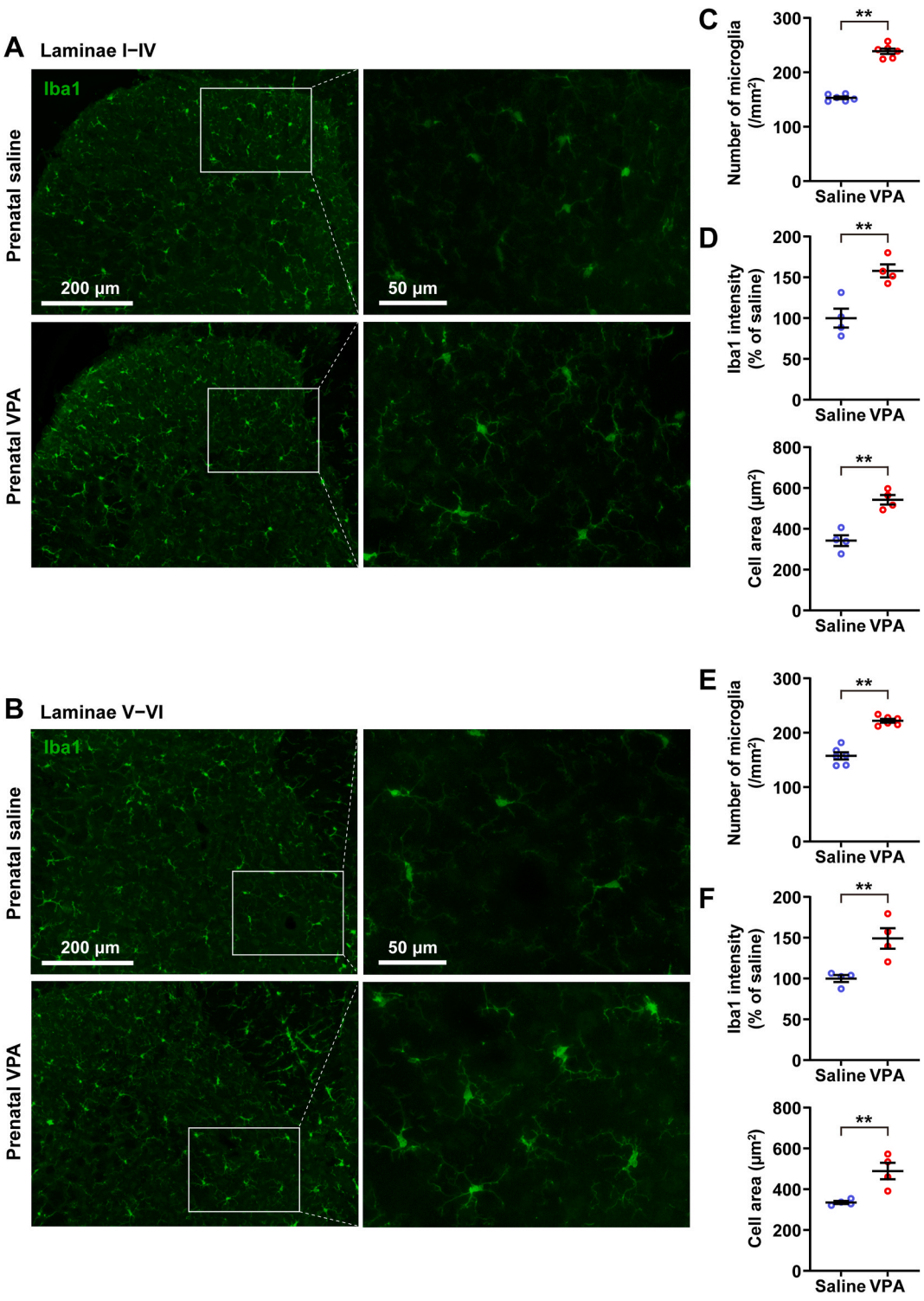

Fig. 2. Increased expression and activation of microglia in the dorsal horn of the spinal cord in prenatally VPA-treated mice. Representative images of Iba1-positive cells in laminae I–IV (A) and V–VI (B) of the dorsal horn of the spinal cord in mice at the age of 8 weeks are shown. Numbers of microglia in laminae I–IV (C) and V–VI (E) were counted. Results are expressed as the mean ± S.E.M. of six mice per group. $^{**}P < 0.01$. Average intensity and cell area per Iba1-labeled microglia in laminae I–IV (D) and V–VI (F) were analyzed. Five microglia were randomly selected per section, and the values were averaged for each animal. Results are expressed as the mean ± S.E.M. of four mice per group. $^{**}P < 0.01$.

and V–VI (Fig. 3B) of the dorsal horn in prenatally VPA-treated mice. Moreover, increased expression of CD68 was detected in microglia in the spinal cord of prenatally VPA-treated mice compared with control mice.

3.3. Changes in microglia morphology in prenatally VPA-treated mice

Microglia are dynamic immune cells of the central nervous system, and their morphology is used as a readout of cellular function (Ziebell et al., 2015). To assess the microglial phenotype, we analyzed the morphology of Iba1-labeled microglia in the spinal cord sections.

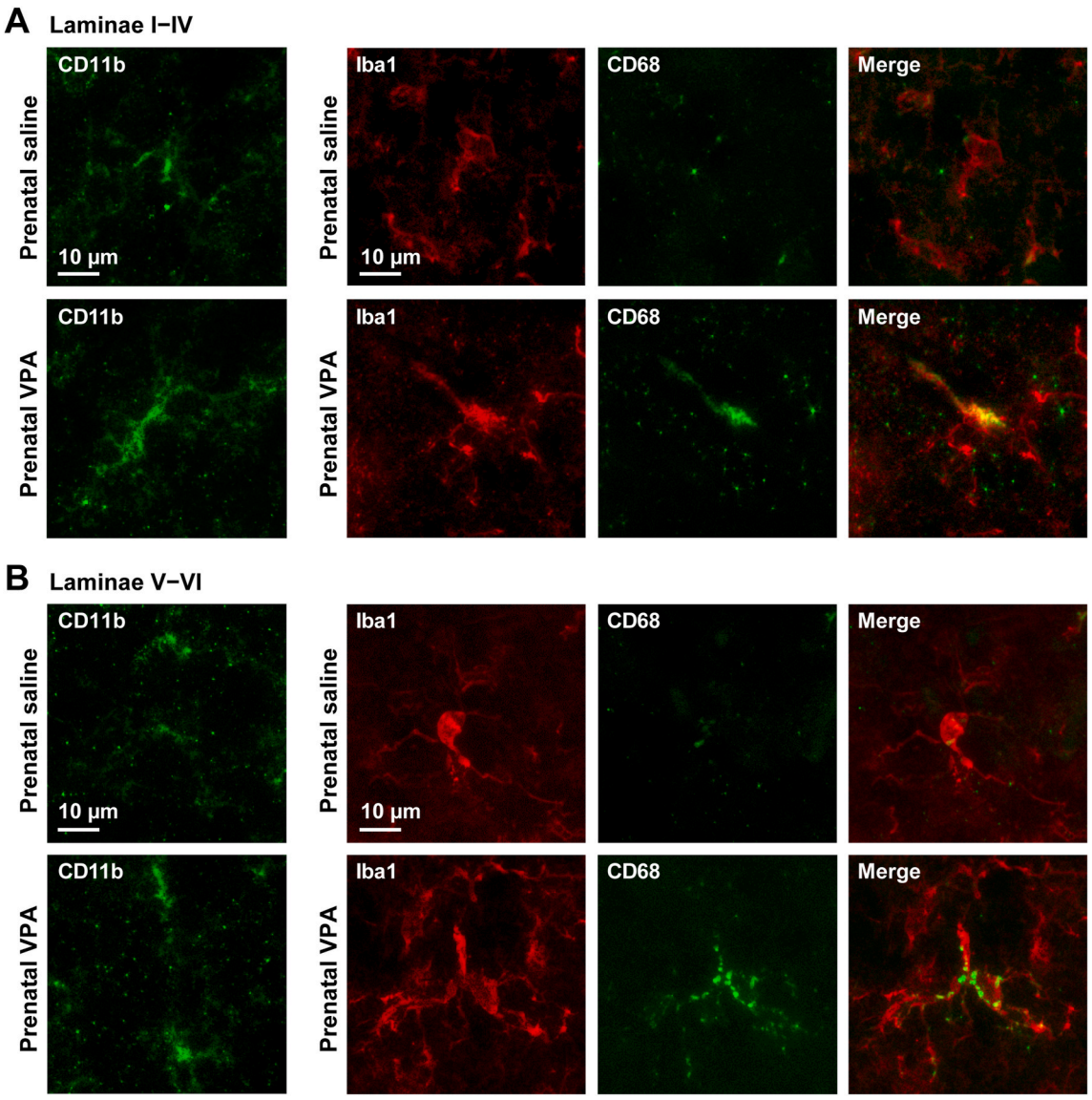

Fig. 3. Increased levels of CD11b and CD68 immunoreactivity in the dorsal horn of the spinal cord in prenatally VPA-treated mice. Representative images of CD11b-positive and Iba1/CD68 double-positive cells in laminae I–IV (A) and V–VI (B) of the dorsal horn of the spinal cord in mice at the age of 8 weeks are shown.

Representative illustrations of microglia by manual tracing methods are shown in Fig. 4. The cell soma size and total number and length of microglial cell processes in laminae I–IV (Fig. 4A) and V–VI (Fig. 4B) in prenatally VPA-treated mice were increased compared with those in control mice, indicating a hyper-ramified morphology.

3.4. Administration of PLX3397 decreased the number of spinal microglia and attenuated mechanical allodynia in prenatally VPA-treated mice

To determine whether microglial reaction contributed to increased pain sensitivity upon prenatal VPA exposure, we applied a CSF1R inhibitor, PLX3397, to adult mice for 10 days to deplete microglia (Fig. 5). Representative images of microglia in laminae I–IV (Fig. 5A) and V–VI (Fig. 5B) of the dorsal horn are shown. We found a significant decrease in the numbers of Iba1-positive cells in PLX3397-treated mice compared with those in mice given control diets, in prenatally VPA- and saline-treated mice. The administration of PLX3397 attenuated mechanical allodynia in prenatally VPA-treated mice (Fig. 5C). Meanwhile, PLX3397 did not affect the withdrawal threshold of prenatally saline-treated mice, possibly due to a ceiling effect.

3.5. Intrathecal injection of Mac-1-saporin abolished mechanical allodynia in prenatally VPA-treated mice

To determine whether spinal microglia are responsible for prenatal VPA exposure-induced pain sensitivity, we depleted spinal microglia in prenatally VPA-treated adult mice by intrathecally injecting Mac-1-saporin, a microglia-selective toxin that was reported to deplete 50% of spinal microglia 1 day after injection (Yao et al., 2016). Representative images of microglia in laminae I–IV (Fig. 6A) and V–VI (Fig. 6B) of the dorsal horn are shown. As expected, 1 day after the intrathecal injection of Mac-1-saporin, the number of microglia was strikingly decreased in the spinal dorsal horn. We depleted 40–50% of spinal microglia by intrathecally injecting Mac-1-saporin in prenatally VPA-treated mice and found that the depletion of microglia in the spinal cord ameliorated mechanical allodynia (Fig. 6C). Although we did not examine the effect of Mac-1-saporin in prenatally saline-treated control mice, a mechanical threshold less than 0.1 g force in prenatally VPA-treated mice was partially recovered by intrathecal injection of Mac-1-saporin (withdrawal threshold; about 0.4 g force) in comparison with prenatally saline-treated mice (more than 0.5 g force, Figs. 1B and 5C).

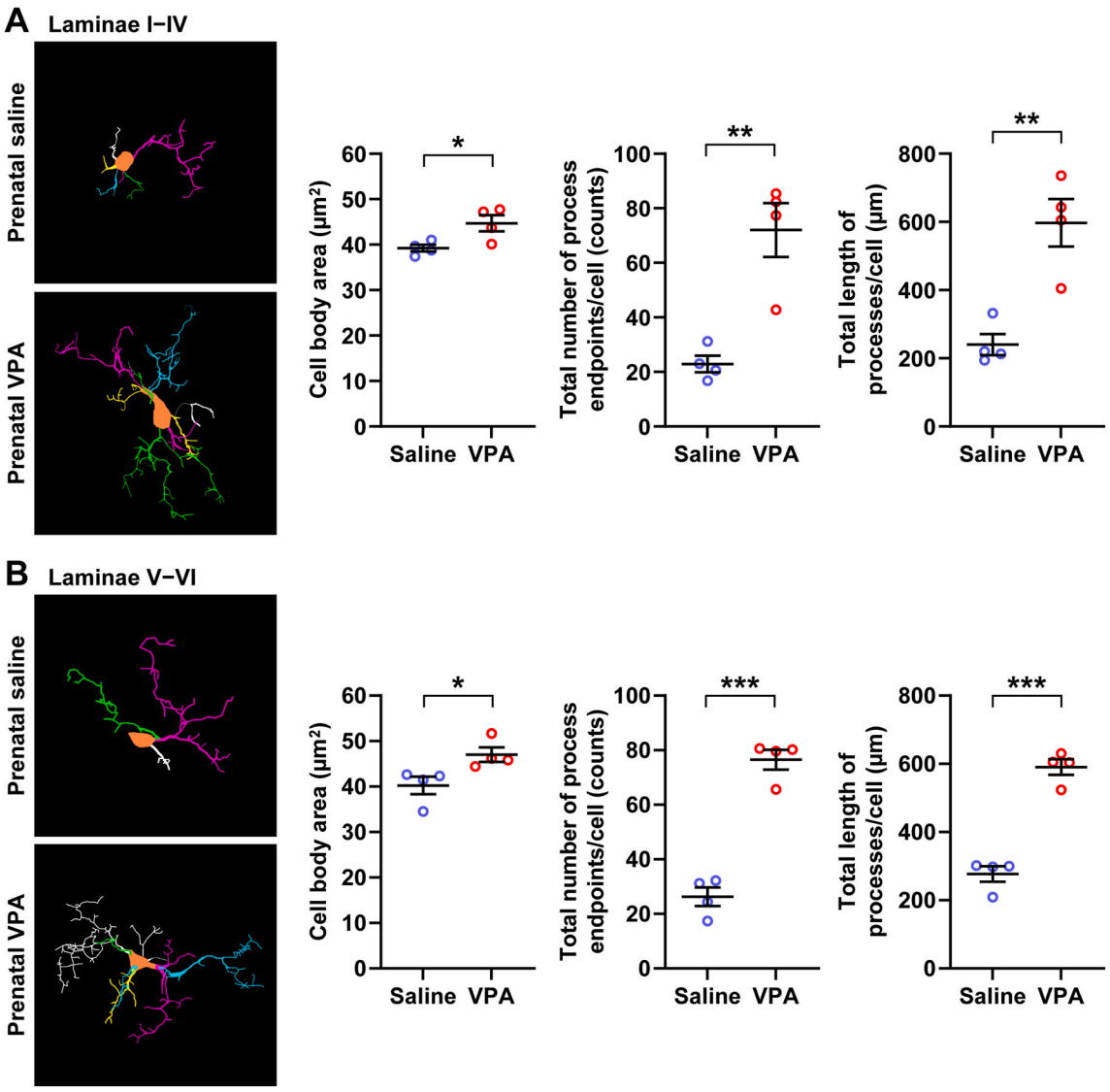

Fig. 4. Changes in microglia morphology in prenatally VPA-treated mice. Digitally reconstructing microglia from high magnification (60 ×) images are shown. The Iba1-labeled microglia cell soma and branches are manually traced and reconstructed by using Neurolucida software. Average cell body area and total number and length of cell processes per Iba1-labeled microglia in laminae I–IV (A) and V–VI (B) were analyzed. Five microglia were randomly selected per section, and the values were averaged for each animal. Results are expressed as the mean ± S.E.M. of four mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

4. Discussion

In the present study, prenatally VPA-treated mice exhibited an increased nociceptive response to mechanical stimuli in juvenile (4-week-old) and adult (8-week-old) periods, indicating a long-lasting behavioral change in response to tactile stimulation. Prenatal VPA-induced mechanical allodynia was also observed in adolescent, but not adult, rats (Schneider et al., 2006). The difference in the effects of prenatal VPA exposure in adulthood might be due to the difference in the experimental conditions, such as species, rearing conditions, and a large variety of behavioral tests applied, but the exact reason is unclear. Additionally, several reports have been published showing that mice with mutations in ASD-related genes, *Shank3* (Orefice et al., 2016), *Fmr1* (He et al., 2017), *Ube3a* (McCoy et al., 2017), and *Mecp2* (Bhattacherjee et al., 2017; Orefice et al., 2016), displayed hypersensitivity to tactile stimuli. Thus, these animals can serve as useful models to elucidate the mechanism behind the abnormal sensitivity to somatosensory stimuli in idiopathic and syndromic forms of ASD.

We found that prenatally VPA-treated mice exhibited an increased number of microglia in the dorsal horn of the spinal cord. Intensities of Iba1 and CD11b, microglial markers, were increased and the cell bodies became enlarged in prenatally VPA-treated mice, indicating microglial activation. Moreover, increased expression of CD68 was detected in Iba1-positive microglia in the spinal cord of prenatally VPA-treated mice compared with control mice. CD68 is a transmembrane protein localized in cellular, lysosomal, and endosomal membranes of monocytes and macrophages/microglia (Jurga et al., 2020). This protein level is known to be upregulated during inflammation (Brown et al., 2021; Calvo and Bennett, 2012; Kiguchi et al., 2018). Interestingly, morphological analysis revealed that prenatal VPA exposure increased microglial cell body area, as well as increasing the number and length of microglial cell processes, indicating a hyper-ramified phenotype. Microglia normally exist in a "resting" state, but can become activated in response to insults. Microglia activation is thought to be a continuum between ramified activated, amoeboid and back again with the first stage in microglial activation typically described as the "alert", hyper-ramified or bushy microglia morphology (Beynon and Walker, 2012; Ladeby et al., 2005; Ziebell et al., 2015). Hyper-ramified microglial activation has been observed in the brain from chronically stressed animals (Crews et al., 2017; Smith et al., 2019). Hyper-ramified microglia retract their

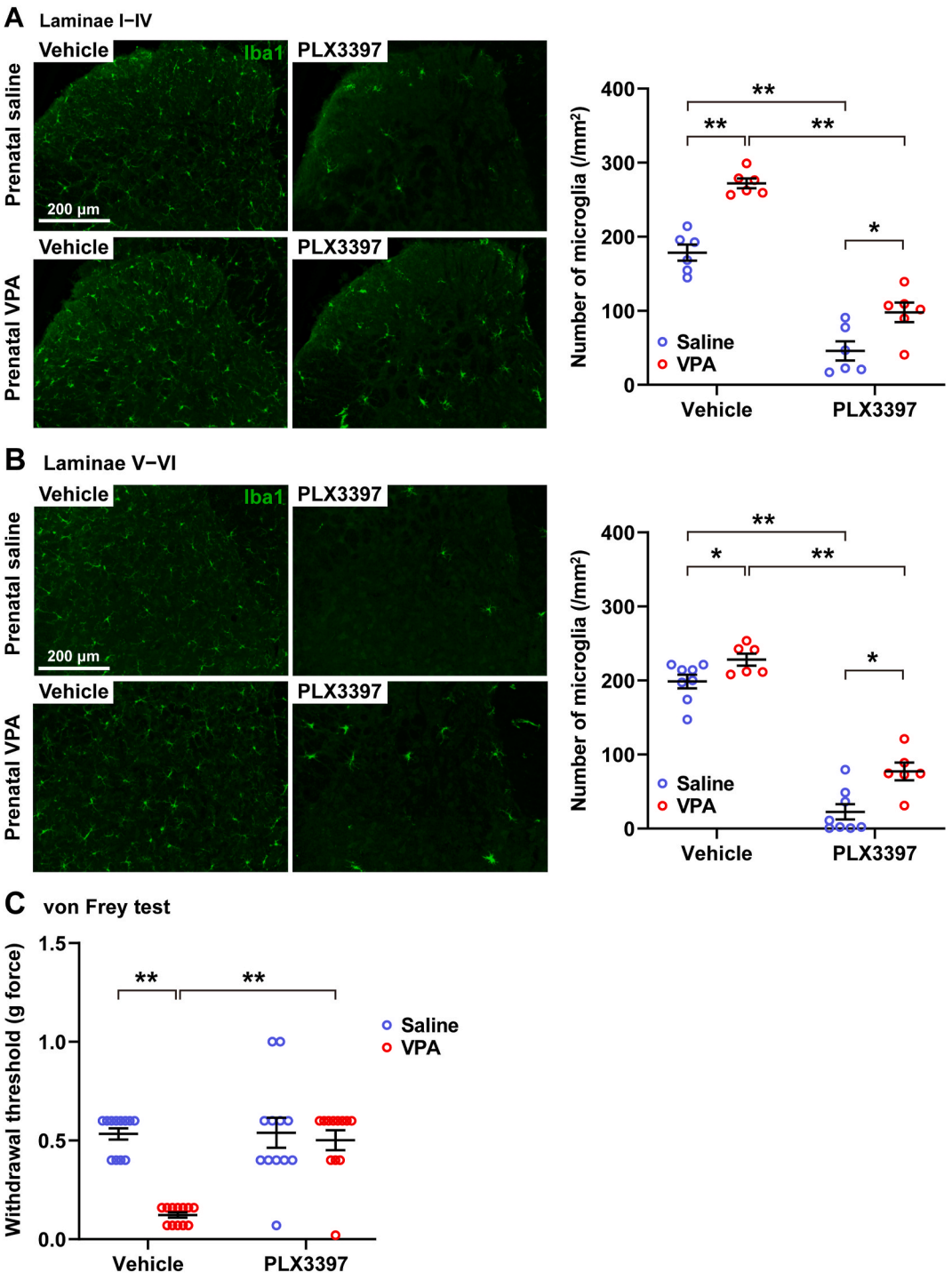

Fig. 5. Effects of PLX3397 on mechanical allodynia in prenatally VPA-treated mice. To deplete microglia in vivo, PLX3397 was orally administered to 8-week-old mice for 10 days. Representative images of microglia in laminae I–IV (A) and V–VI (B) of the dorsal horn are shown. Numbers of Iba1-positive microglia in laminae I–IV and V–VI were counted. Results are expressed as the mean ± S.E.M. of six mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$. (C) The von Frey test was performed to assess the sensitivity to tactile stimuli. Results are expressed as the mean ± S.E.M. of 12 mice per group. $^{**}P < 0.01$.

processes from the surrounding tissue and thicken, acquiring a "bushy-like" appearance and display immunoreactivity for CD11b, suggesting that these cells have a functional inflammatory role. The exact relationship between the morphological and functional activation states is currently unclear. Future studies are needed to reconcile the hyper-ramified microglial cell morphology observed here with the increased inflammatory status.

Spinal microglial activation has long been considered a significant contributor to neuropathic pain after peripheral nerve injury (Tsuda, 2019; Tsuda et al., 2005; Watkins et al., 2001; Nakamura et al., 2021). To further explore the involvement of the microglial reaction in prenatal VPA treatment-induced mechanical allodynia, microglia were depleted by administering PLX3397 (Saika et al., 2021; Elmore et al., 2014). Treatment with PLX3397 induced a robust reduction in microglia number and the elimination of most microglia in both prenatally VPA-treated and vehicle-treated control mice. Here, we observed that the depletion of microglia attenuated mechanical allodynia in prenatally VPA-treated mice, but not in control mice. Furthermore, we depleted

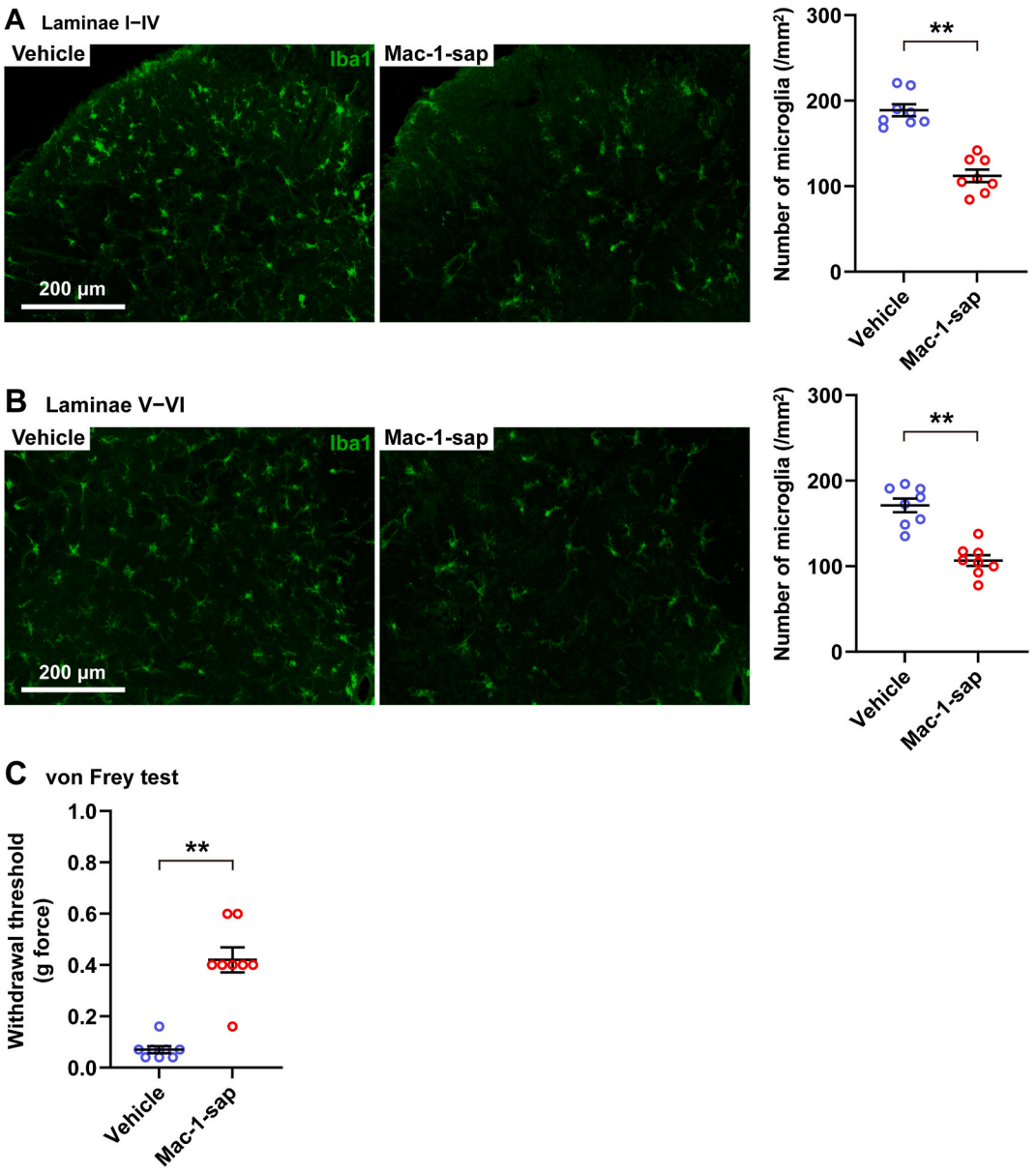

Fig. 6. Effects of intrathecal injection of Mac-1-saporin on mechanical allodynia in prenatally VPA-treated mice. To deplete microglia in the spinal cord, Mac-1-saporin (Mac-1-sap; 11.2 μg/5.5 μl) was injected intrathecally at the level of L4–L5 in prenatally VPA-treated mice at the age of 8 weeks. Vehicle groups were injected with the same volume of saline. Representative images of microglia in laminae I–IV (A) and V–VI (B) of the dorsal horn are shown. Numbers of Iba1-positive microglia in laminae I–IV and V–VI were counted. Results are expressed as the mean ± S.E.M. of eight mice per group. $^{**}P < 0.01$. (C) The von Frey test was performed to assess the sensitivity to tactile stimuli. Results are expressed as the mean ± S.E.M. of eight mice per group. $^{**}P < 0.01$.

spinal microglia by intrathecally injecting Mac-1-saporin (Yao et al., 2016) in prenatally VPA-treated mice and found that the depletion of microglia in the spinal cord also ameliorated mechanical allodynia. Together, these findings suggest that spinal microglia reaction contributes at least in part to the exacerbated or persistent allodynia induced by prenatal exposure to VPA, but we cannot rule out the influence of brain microglia.

In contrast to the proliferative and activating effects of prenatal VPA exposure on microglia in the brain (de Leão et al., 2021; Dos Santos et al., 2021; Gifford et al., 2022; Traetta et al., 2021) and spinal cord (present study), postnatal treatment with VPA instead suppresses spinal microgliosis and exhibits anti-inflammatory and neuroprotective effects in several in vivo models of neuropathic pain and spinal cord injury (Lu et al., 2013; Chen et al., 2018; Guo et al., 2021). A high dose of VPA acts as a histone deacetylase (HDAC) inhibitor (Göttlicher et al., 2001), and treatment with sodium butyrate, an HDAC inhibitor, is also known to attenuate the proliferation of the murine BV2 microglial cell line (Baby et al., 2014). We previously reported that prenatal VPA exposure at E12.5 induces social interaction deficits in male mice (Kataoka et al., 2013; Kawase et al., 2019; Hara et al., 2016, 2017; Yamaguchi et al., 2017). Interestingly, chronic treatment with VPA or sodium butyrate in adult VPA-exposed offspring reversed prenatal VPA-induced deficits in social interaction, suggesting that postnatal HDAC inhibition could restore the ASD-related symptoms (Takuma et al., 2014). Thus, it is likely that the effects of VPA differ between prenatal and postnatal stages, although the detailed mechanisms remain unclear.

In this study, an increased number of activated microglia was observed throughout the spinal dorsal horn in prenatally VPA-treated mice. The dorsal horn is divided into six layers, referred to as laminae, and has been recognized as a key site for somatosensory processing (Alles and Smith, 2018; Harding et al., 2020). Primary afferent fibers have been classified by conduction velocity and degree of myelination

into four types (Aα, Aβ, Aδ, and C). Tactile and innocuous information is basically carried by Aβ fibers, which synapse primarily onto dorsal horn neurons in laminae III and IV (Abraira et al., 2017). Additionally, Aδ fibers transmit a mixture of noxious and innocuous tactile and cold information and terminate predominantly within laminae I and V, with a subset of Aδ fibers corresponding to low-threshold mechanosensation terminating within lamina III (Koch et al., 2018; Li et al., 2011; Arcourt et al., 2017). The anomalous distribution of tactile information is reinforced by increased excitatory transmission between deep and superficial laminae. In prenatally VPA-treated mice, the roles of spinal microglia in the somatosensory pathways remain unclear. Further studies are needed to clarify how the circuitry of the dorsal horn processes and modulates somatosensory information. A growing body of evidence indicates that astrocytes have also crucial roles in neuropathic pain induced by peripheral nerve injury (Miranpuri et al., 2021; Thakur et al., 2017) and astrocytes are known to modulate the microglial function (Matejuk and Ransohoff, 2020; Liddelow et al., 2020). Previous studies have shown that prenatal VPA treatment causes reactive gliosis such as increased glial fibrillary acidic protein immunoreactivity in the prefrontal cortex and hippocampus of rats (Codagnone et al., 2015; Elnahas et al., 2022; Traetta et al., 2021), but it is still unclear whether astrocytes in the spinal cord are altered in prenatally VPA-exposed animals. To better understand the pathophysiological mechanism underlying abnormal tactile sensitivity in ASD, it might be important to clarify the role of spinal astrocytes in prenatal VPA-treated mice.

In conclusion, this study demonstrates that prenatal exposure to VPA in mice induces long-lasting mechanical allodynia and microglial activation in the spinal cord, as characterized by increased proliferation and changes in hypertrophic and hyper-ramified cell morphology. Spinal microglia are involved at least in part in the maintenance of allodynia in prenatally VPA-treated adult mice. These findings provide insights into the altered tactile sensitivity of a prenatal VPA-induced model of ASD and may contribute to understanding the mechanism underlying sensory abnormalities and the etiology of ASD. Currently, the detailed mechanism underlying spinal microglial activation by prenatal VPA exposure remains unclear. In the preliminary experiments, we found that prenatal exposure to trichostatin A, an HDAC inhibitor, caused mechanical allodynia in mice. Thus, an HDAC inhibition by a high dose of VPA might be involved in induction of allodynia. Further studies are required to clarify how prenatal VPA treatment causes microglia activation and altered pain sensitivity.

Author contributions

Y.A. conceived and designed the study. E.I., T.K., S.A., M.I., and Y.A. acquired funding. E.I., S.S., A.R.A., T.T., T.K., T.N.B.H., S.H., and Y.A. performed the experiments. E.I., S.S., S.A., and Y.A. analyzed the data. Y. N., K.H.-N., N.M., and N.K. supervised the behavioral experiments. Y.K., M.I., Kazuhiro Tsuga, Kazuhiro Takuma, N.M., and N.K. supervised the study. E.I. and Y.A. wrote the manuscript. All authors reviewed and approved the manuscript.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2022.105415.

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