Brain, Behavior, and Immunity 90 (2020) 319–331

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Early life stress from allergic dermatitis causes depressive-like behaviors in adolescent male mice through neuroinflammatory priming

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ARTICLE INFO

Keywords:
Neuroinflammation
Early life stress
Allergic dermatitis
Depression
Neuroinflammatory priming
Microglia
Abnormal kynurenine metabolism

ABSTRACT

Allergic dermatitis (AD), associated with pruritus and itchiness, is one of the major stressful conditions early in life. AD also influences the incidence of neuropsychiatric disorders and developmental disorders through neuroimmune interactions. To the best of our knowledge, there is no report that assesses the effects of early childhood dermatitis on psychiatric disorders later in life using an animal model. Here, we developed an oxazolone (Ox)-induced AD model in the early life of male C57BL/6J mice whose ears were challenged by Ox repeatedly from postnatal days (PD) 2 to PD30. On PD30, the Ox-treated ears were remarkably thickened and showed epidermal hyperplasia coupled with increased expression of T helper 2 cytokines, *interleukin (IL)-4*, and *IL-13* in the ear tissue. Additionally, serum immunoglobulin E levels and serum corticosterone levels were higher in the Ox-treated mice than those in the control mice. Although Ox-treated PD40 mice showed neither behavioral abnormalities nor increases in pro-inflammatory cytokine expression in the brain, this study revealed that they experienced downregulation of *CD200R1* expression in the amygdala under basal conditions and that additional lipopolysaccharide (LPS) administration induced enhanced neuroinflammatory reaction as the priming effect was accompanied by an increase of Iba-1-positive microglia in the amygdala and hippocampus. Furthermore, the Ox-treated PD40 mice showed depressive-like behaviors 24 h after LPS administration, whereas the control mice did not. Interestingly, the expression of indoleamine 2,3-dioxygenase and kynurenine 3-monooxygenase, key rate-limiting enzymes of the kynurenine metabolism pathway, was upregulated in the hippocampus, prefrontal cortex, and amygdala of the Ox-treated mice 4 h after LPS administration. Based on these results, we suggest that early life stress from AD aggravates susceptibility to systemic inflammation in the adolescent brain, leading to depressive behaviors with abnormal kynurenine metabolism.

1. Introduction

The prevalence of allergic conditions, including atopic dermatitis, asthma bronchitis, and allergic rhinitis, has largely increased worldwide, specifically in developed countries (Lau et al., 2019). Atopic dermatitis (AD) is a chronic inflammatory skin disease typically exhibiting childhood onset, which impairs the patient's quality of life as this disease results in intense pruritus stress. It is now revealed that AD is an initial manifestation of subsequent allergic disorders, known as atopic march, defined as the natural history of atopic conditions. Generally, AD in infancy or early childhood predates the development of other allergic disorders experienced later in childhood (Dharmage et al., 2014). Although the pathogenesis of AD has not been fully elucidated so far, it is believed that complex interactions among the

Abbreviation: AD, atopic dermatitis; ADHD, attention-deficient/hyperactivity disorder; EtOH, ethanol; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HPA, hypothalamic-pituitaryadrenal; Iba-1, Ionized calcium binding adaptor molecule 1; IDO, indoleamine 2,3-dioxygenase; INF, interferon; Ig, immunoglobulin; IL, interleukin; KMO, kynurenine 3-monooxygenase; KP, kynurenine pathway; LPS, lipopolysaccharide; MCP, monocyte chemotactic protein; NF, nuclear factor; Ox, oxazolone; OFT, open-field test; PFC, prefrontal cortex; PD, postnatal day; QA, quinolinic acid; RANTES, Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted, normal T-cell expressed and secreted; RT-PCR, reverse transcription polymerase chain reaction; SEM, standard error of the mean; SPT, sucrose preference test; STAT, signal transducers and activators of transcription; TGF, transforming growth factor; TLR4, Toll-like receptor 4; Th, T helper; TNF, tumor necrosis factor; TST, tail suspension test

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https://doi.org/10.1016/j.bbi.2020.09.013
Received 17 June 2020; Received in revised form 10 September 2020; Accepted 13 September 2020
Available online 17 September 2020

susceptible genes, host's environment, and skin barrier dysfunction result in the activation of inflammatory pathways where a T helper 2 (Th2) cell response and an elevated immunoglobulin E (IgE) response to antigens are dominant (Sacotte and Silverberg, 2018).

More importantly, AD has a significant impact not only on subsequent allergic complications but also on neuropsychiatric disorders, including depression, anxiety, attention-deficit/hyperactivity disorder (ADHD), and autism (Yaghmaie et al., 2013). Neuropsychiatric disorders are some of the leading causes of disorders worldwide, and approximately 10% of the world's population is experiencing depression or anxiety associated with increased morbidity and mortality (Global Burden of Disease Study, 2015). Although several researchers recently reported that there was a significant association between AD and neuropsychiatric disorders in both children and adults (Schonmann et al., 2020; Rønnstad et al., 2018; Xie et al., 2019), the mechanism of the association between AD and neuropsychiatric disorders has hardly been investigated. In allergen-induced allergic rodent models, neuroinflammation in the hippocampus with microglial activation has been shown to be associated with behavioral abnormalities (Yang et al., 2018; Klein et al., 2016; Germundson et al., 2018). In another allergic rodent model, allergen-sensitized animals showed anxiety-like behaviors with the activation of microglia and astrocytes in the prefrontal cortex (PFC) and amygdala (Dehdar et al., 2019). More recently, one group reported that AD-like adult mice showed behavioral abnormalities associated with aberrant dopamine reward circuitry (Yeom et al., 2020).

It has been revealed that neuroinflammation is an underlying pathogenesis of neuropsychiatric disorders, including depression, ADHD, autism, and neurodegenerative disorders (Mattei and Notter, 2020; Nakagawa and Chiba, 2016; DiSabato et al., 2016). Microglia, a resident innate immune cell within the brain, is a key player in neuroinflammation and is well known not only to act in the brain's defense responses to adverse stimuli but also to modify neuronal networks contributing to neuronal activity and synaptic plasticity (Wohleb, 2016). Several studies have reported that chronic stress evoked microglial activation, producing pro-inflammatory cytokines, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α in the limbic system and PFC, where intensive neural connections exist, leading to psychiatric disorders (Johnson et al., 2019; Mondelli and Vernon, 2019). The concept of microglial priming is derived from a macrophage property that enhances immune responsiveness induced after an initial inflammatory stimulation (Neher and Cunningham, 2019). In both acute and chronic stress conditions, primed microglia acquire a potentiated pro-inflammatory cytokine response to consecutive lipopolysaccharide (LPS) stimulation after an initial insult, resulting in behavioral abnormalities (Frank et al., 2007; Nava Catorce and Gevorkian, 2016).

The present study aimed to first develop an allergic dermatitis animal model during the early life period using C57BL/6J male mice, since there is no report on the effects of early life dermatitis on mental health in adolescents. We verified the condition of allergic dermatitis in our model mice and subsequently assessed behavioral changes and pro-inflammatory cytokine expression within the limbic system and PFC regions. Additionally, we tested the hypothesis that allergic dermatitis-affected animals would be more susceptible to a second insult with LPS administration compared to allergic dermatitis-unaffected animals and postulated that systemic inflammation would reveal the effects of early life dermatitis more clearly.

2. Methods

2.1. Animals

Pregnant female C57BL/6J mice were purchased from Charles River Laboratories on gestational day 14. Each pregnant mouse was housed individually in a temperature- and humidity-controlled room with food and water as desired. All experimental procedures were conducted in strict accordance with the regulations of the National Institute of Neuroscience (Tokyo, Japan) for animal experiments and were approved by the Institutional Animal Investigation Committee (approval number: 2018024). All efforts were made to minimize animal suffering and reduce the number of animals used. All mice were housed at the Animal Centre of the National Institute of Neuroscience, National Center of Neurology and Psychiatry (Tokyo, Japan).

2.2. Oxazolone (Ox)-induced dermatitis

After parturition (designated as PD0), PD1–2 litters were randomly culled to a total of 6–8 pups per dam and then each litter was randomly divided into three subgroups as previously reported (Koppensteiner et al., 2014); one group was left untreated as the naïve group in their home cage, except for routine cage changes. The second group was treated with 10 μL of ethanol (EtOH) applied to both the flanks and ear buds as the control group on PD2. The third group was sensitized by a single topical treatment with 10 μL of 3% oxazolone (Ox) in EtOH to both the flanks and ear buds on PD2. A week later, the control and Ox-treated groups were topically treated with 10 μL of EtOH and 1% Ox on both ears, respectively. We administered the treatment for a total of ten times, once every 2 or 3 days for an additional 3 weeks. The pups in each group were weaned at PD24, and 3–4 animals were weaned in a cage until they were utilized at PD40. Considering hormonal effects on neuronal functions, only male mice were selected for this study. The ear thickness was measured using a dial thickness gauge (Mitsutoyo Co., Tokyo, Japan), and ear and brain tissue samples were collected on PD30. On PD40, adolescent mice were examined for basal measurements or administered LPS (1 mg/kg body weight) intraperitoneally and later sacrificed 4 or 26 h after behavioral examinations. All tests were conducted during the light phase of the illumination cycle. Animals in the experimental and control groups were tested alternately to avoid diurnal variation bias.

2.3. Blood and tissue preparation

After the mice were deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), blood samples were collected by cardiac puncture for measurement of serum IgE and cytokines. Subsequently, their brains were rapidly removed, placed on ice, and sliced into 1-mm thick slices using the Rodent Brain Matrices (Electron Microscopy Sciences, Hatfield, PA, USA). Slices with areas of interest (the hypothalamus, hippocampus, PFC, and amygdala) were separately isolated according to a brain atlas.

2.4. Histological and immunohistochemical analyses

After blood sampling, each mouse was transcardially perfused with phosphate-buffered saline and 4% paraformaldehyde. The brain was removed from the skull cavity, the ear was cut off, and the sample post-fixed overnight in 4% paraformaldehyde. Coronal slices (20-μm thickness) were prepared using a vibratome. Slices were selected according to a brain atlas and incubated in PBS containing 3% normal goat serum (Vector Laboratories, Burlingame, CA, USA) and 0.3% Triton X-100 for 1 h at room temperature. They were then treated with anti-ionized calcium-binding adaptor molecule-1 (Iba-1) antibodies (1:5000 dilution; Wako, Osaka, Japan) overnight at 4 °C in PBS and washed with PBS. The signals were visualized using the avidin-biotin-peroxidase complex method (Vector Laboratories, Burlingame, CA, USA), as previously described (Yamada et al., 2017). Hematoxylin and eosin staining was performed as previously reported (Kikuchi et al., 1990).

Photomicrographs of sections were taken using a BZ-X710 All-in-One Microscope (Keyence, Tokyo, Japan) with a 20× objective lens. Iba-1-positive cells were counted with the threshold method (Beynon and Walker, 2012; Spencer et al., 2019), using ImageJ software ver. 1.53 (National Institutes of Health, Bethesda, MD, USA). In brief, the

thresholding procedure was performed by adjusting the pixels of the image to be included in the quantification to those that encompass the staining of interest and not the background staining. The threshold intensity for each brain region was determined by adjusting the optimal threshold manually for each animal in the naïve group for each region as the control. The optimal threshold was then applied to all the sections assessed for each animal in all groups and the number of Iba-1-positive cells met the threshold was counted automatically with ImageJ software. The mean number of Iba-1-positive cells counted in three independent high-power fields (200× field) from nine sections per brain region was used.

2.5. Lipopolysaccharide (LPS) challenge

On PD40, adolescent mice were administered with LPS (1 mg/kg body weight, reconstituted with sterile saline) (L2018; *Escherichia coli* strain K-235; Sigma-Aldrich, St. Louis, MO, USA) or sterile saline intraperitoneally. The dosage of LPS adapted in this study was such that it would cause the upregulation of central cytokines and induce sickness without a lethal effect in normal mice (O'Connor et al., 2009). After LPS administration, locomotor activity was examined 4 h later, and behavioral tests (see below) and sucrose preference, open-field, and tail suspension tests were performed 24 h later. For analysis of gene expression, the mice were sacrificed 4 or 26 h after LPS administration.

2.6. Behavioral tests

We conducted all behavioral tests during the light phase of the illumination cycle. On the test day, the mice were transported to the testing room and left in their home cages for at least 1 h before the test.

2.6.1. Locomotor activity

To assess locomotor activity after LPS administration, the mice were individually placed in a clean novel cage, similar to the home cage. Locomotor activity in a novel cage was calculated automatically using a software (TimeOFCR, O'Hara, Tokyo, Japan).

2.6.2. Open-field test

The open-field test was performed as previously described (Zushida et al., 2007). To start each session, the mice were placed at the peripheral corner of the open-field arena (50 × 50 cm white field surrounded by a 40-cm-high white wall and illuminated with 10 lx) (O'Hara, Tokyo, Japan) and allowed to explore for 5 min. The test sessions were recorded by a video camera above the arena. Locomotor activity and the time spent in the center area (30 cm × 30 cm square) were automatically analyzed using a software (TimeOFCR, O'Hara, Tokyo, Japan).

2.6.3. Tail suspension test

Tail suspension test was performed as previously described (Kuniishi et al., 2020). In the tail suspension test, the mice were subjected to a 6-min session each, wherein a mouse was suspended by its tail with an adhesive tape to an aluminum bar, and the duration of immobility was measured. Clear hollow cylinders cut from polycarbonate tubing (4 cm in length, 1.6 cm outside diameter, 1.2 cm inside diameter, 3.8 g) were placed around the tails of the mice to prevent tail-climbing behavior. The mice were judged to be immobile when they remained motionless, except for whisker movement and respiration.

2.6.4. Sucrose preference test

The sucrose preference test (SPT) was conducted for 3 days, including 1 day for the training period, with some modifications from previously established conditions (Nakatake et al., 2019; Mao et al., 2014; Cordeiro et al., 2019). Mice were trained to drink from two separate bottles (water and 1% sucrose) for 1 day. The two drinking bottles were located on both sides of the home cage of the experimental mice. The positions of the two bottles were balanced across the experimental mice to exclude potential side preference bias. On the second day of SPT, the mice were deprived of food, water, and sucrose for 24 h. After the deprivation, they were again provided access to water and sucrose bottles, and their total liquid consumption was recorded for 1 h to obtain sucrose preference. Sucrose preference was calculated by dividing sucrose consumption by the total consumption (sucrose + water).

2.7. RNA extraction and quantitative reverse transcription polymerase chain reaction

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The primer sequences are shown in Supplementary Table S1. Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) in a CFX96 Real-Time PCR Cycler (Bio-Rad, Hercules, CA, USA). The threshold cycle (Ct) values were determined by plotting the observed fluorescence against the cycle number. Ct values were analyzed using the comparative Ct method and normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The relative gene expression levels were estimated using the following formula: relative expression $= 2^{-(Ct[\text{target gene}] - Ct[GAPDH])}$.

2.8. Corticosterone assay

Considering the circadian rhythm of serum corticosterone level, the trunk blood was quickly collected after decapitation in the morning (8:00–9:00 am) without anesthesia only for measurement of serum corticosterone. The serum was collected and stored at −80 °C prior to analysis. Serum corticosterone levels were determined using a corticosterone enzyme-linked immunosorbent assay kit (Enzo Life Sciences, New York, USA) according to the manufacturer's instructions.

2.9. Serum IgE and cytokine assays

Serum levels of IgE (IgE ELISA test kits, Yamasa Co. Ltd., Chiba, Japan) and the cytokines IL-1β, IL-6, and TNFα (Quantikine murine kits, R&D Systems, Minneapolis, MN, USA) were measured using enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions.

2.10. Statistical analyses

All data are expressed as mean ± standard error of the mean. Data were analyzed by one-way or two-way analysis of variance (ANOVA), as appropriate. Two-way ANOVA was performed with the treatment (Naïve, EtOH, Ox) and LPS administration (saline, LPS) as the between-subject variables to reveal significant main factor effects or interactions. Post-hoc analysis of group differences was performed with the Turkey honest significant difference test. A p-value < 0.05 was considered significant. All statistical analyses were performed using the statistical software R version 3.5.3 (R Core Team, 2019).

3. Results

3.1. Repeated Ox treatments resulted in remarkable epidermal hyperplasia in the ear tissue in adolescent male mice, showing body weight loss under stress conditions

To establish an allergic dermatitis model of the early life of mice, PD2 neonatal pups were sensitized with 3% Ox treatment. Subsequently, ten 1% Ox challenges were applied to both ears until PD30 (Fig. 1A). After multiple Ox treatments, apparent hyperplasia on both ears was observed in the Ox-treated mice due to scratching

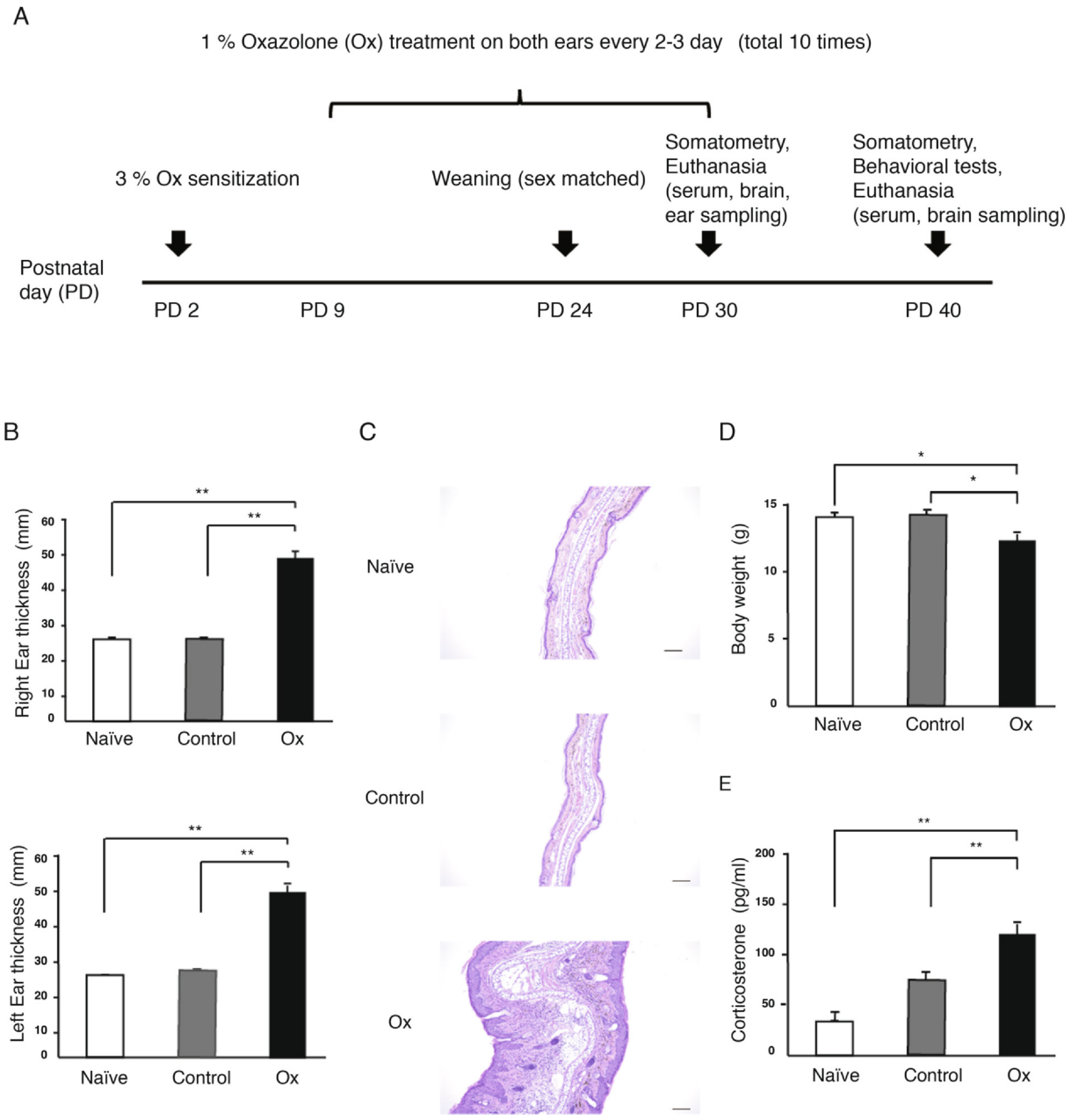


Fig. 1. Characterization of oxazolone (Ox)-induced allergic dermatitis model in early life mice. (A) Representation of the experimental schedule for Ox-induced dermatitis. (B) Ear thickness measurement on postnatal day (PD) 30 after ten Ox challenges (right ear [one-way ANOVA: $F(2,45) = 129.9$, $P < 0.01$; Naïve n = 15, Control n = 17, Ox n = 16], left ear [one-way ANOVA: $F(2,45) = 111.5$, $P < 0.01$; Naïve n = 15, Control n = 17, Ox n = 16]). (C) Hematoxylin and eosin staining of ear tissues was performed on PD30 (scale bar, 50 μm). Original magnification: 100 × (D) Body weight in each group was measured on PD30 [one-way ANOVA: $F(2,45) = 5.21$, $P < 0.01$; Naïve n = 15, Control n = 17, Ox n = 16]. (E) Serum corticosterone levels were measured in the morning (8:00–9:00 am) of PD30 after 10 Ox challenges [one-way ANOVA: $F(2,21) = 17.51$, $P < 0.01$; Naïve n = 8, Control n = 8, Ox n = 8]. Values are expressed as mean ± standard error of the mean ($^{*}p < 0.05$, $^{**}p < 0.01$).

behavior (Fig. 1B). Histological analysis revealed that the ear tissues in both the naïve and control mice were normal, whereas epidermal hypertrophy, hyperkeratosis, and infiltration of inflammatory cells were observed in the ear tissues of the Ox-treated mice on PD30 (Fig. 1C). Additionally, the body weight of the Ox-treated mice was significantly lower compared to that of untreated and control mice. Moreover, serum levels of the stress hormone, corticosterone, were increased in the Ox-treated male mice on PD30 (Fig. 1D, 1E).

3.2. Atopic dermatitis-like features, T helper 2 dominant induction within a lesion, and increased serum immunoglobulin E level were confirmed in the Ox-treated mice on PD30

We next assessed the gene expression patterns of inflammatory cytokines and chemokines involved in AD in the hyperplastic lesions of the Ox-treated mice on PD30. As shown in Fig. 2A, the Ox-treated ear tissue exhibited strong expression of *IL-4* and *IL-13*, known as Th2 cytokines, whereas the expressions of *interferon-γ* and *IL-12*, known as Th1 cytokines, were comparable to those of control ear tissue. Although there was no change in the expression levels of other AD-related cytokines, *IL-17* and *IL-10*, the expression levels of the allergic-related

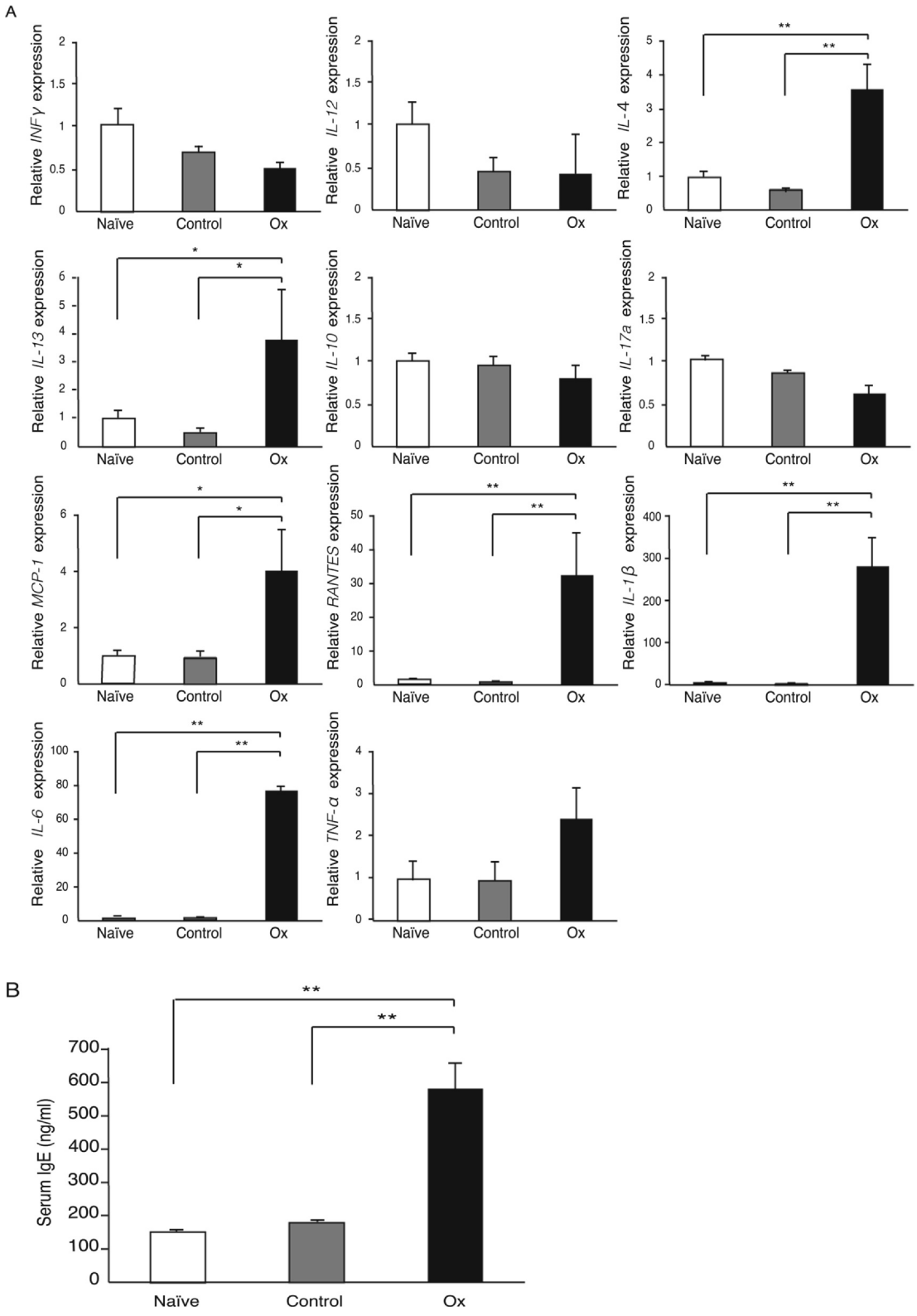


Fig. 2. Induction of allergic properties in oxazolone (Ox)-treated mice. (A) Expression patterns of cytokines and chemokines involved in the allergic condition analyzed by quantitative reverse transcription polymerase chain reaction of ear samples of postnatal day (PD)30 mice, normalized to glyceraldehyde 3-phosphate dehydrogenase expression (*INF*γ [one-way ANOVA: $F(2,21) = 4.78$, $P < 0.05$], *IL-12* [one-way ANOVA: $F(2,21) = 2.49$, $P = 0.11$], *IL-4* [one-way ANOVA: $F(2,21) = 12.1$, $P < 0.01$], *IL-13* [one-way ANOVA: $F(2,21) = 4.78$, $P < 0.05$], *IL-10* [one-way ANOVA: $F(2,21) = 0.72$, $P = 0.50$], *IL-17a* [one-way ANOVA: $F(2,21) = 2.94$, $P = 0.07$], *MCP-1* [one-way ANOVA: $F(2,21) = 4.33$, $P < 0.01$], *RANTES* [one-way ANOVA: $F(2,21) = 6.05$, $P < 0.01$], *IL-1β* [one-way ANOVA: $F(2,21) = 16.97$, $P < 0.01$], *IL-6* [one-way ANOVA: $F(2,21) = 10.43$, $P < 0.01$], *TNFα* [one-way ANOVA: $F(2,21) = 1.97$, $P = 0.20$]) (Naïve n = 7, Control n = 9, Ox n = 8). (B) Serum immunoglobulin E level measured on PD30 mice after 10 Ox challenges [one-way ANOVA: $F(2,21) = 18.98$, $P < 0.01$; Naïve n = 8, Control n = 8, Ox n = 8]. Values are expressed as mean ± standard error of the mean ($^{*}p < 0.05$, $^{**}p < 0.01$).

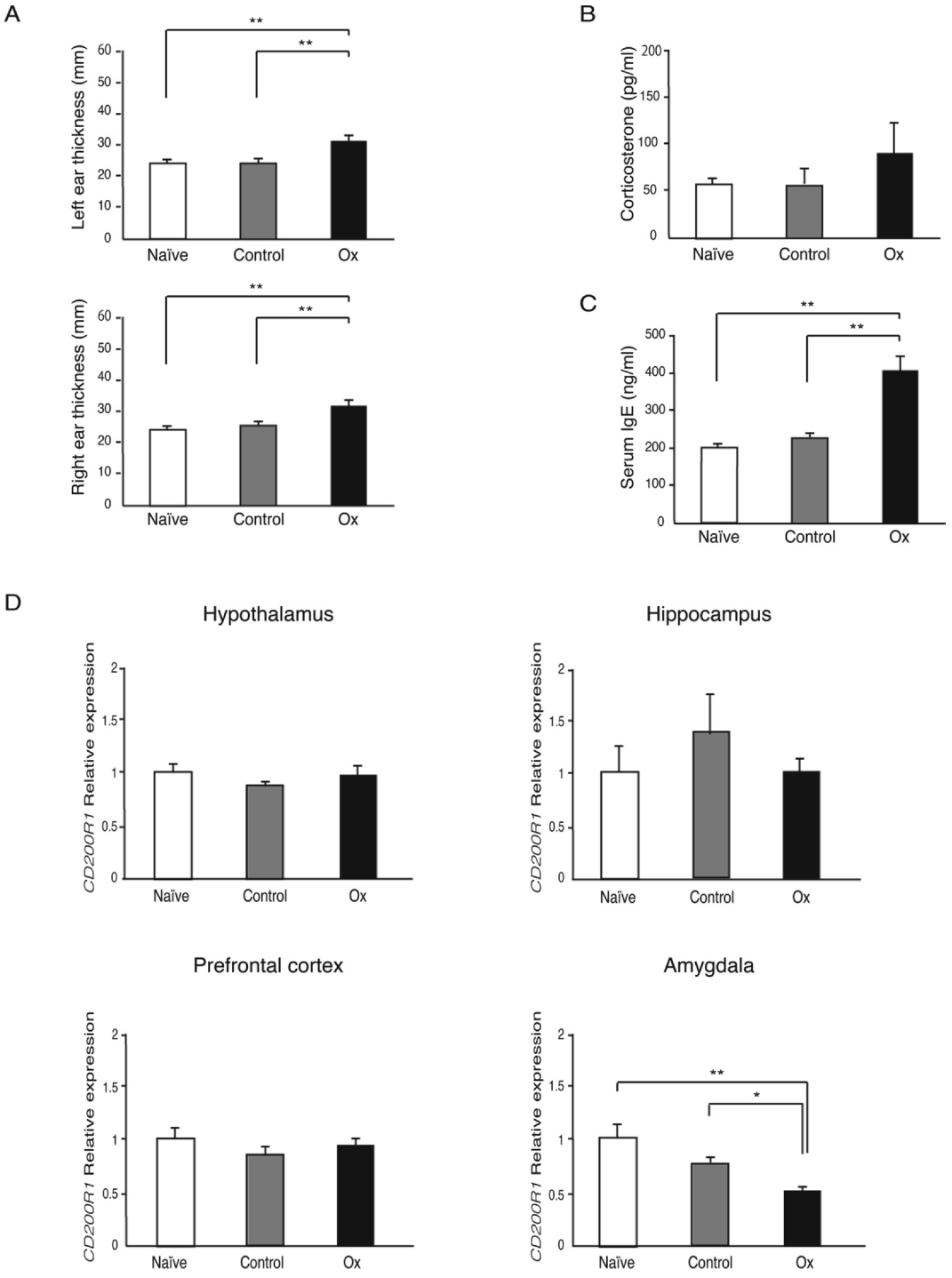


Fig. 3. Characterization of postnatal day (PD)40 adolescent mice 10 days after the last oxazolone treatment. (A) Ear thickness was measured on PD40 in each group (right ear [one-way ANOVA: $F(2,18) = 62.83$, $P < 0.01$; Naïve n = 7, Control n = 8, Ox n = 9], left ear [one-way ANOVA: $F(2,18) = 53.71$, $P < 0.01$; Naïve n = 7, Control n = 8, Ox n = 9]). (B) Serum corticosterone levels were measured in the morning (8:00–9:00 am) of PD40 [one-way ANOVA: $F(2,24) = 0.61$, $P = 0.53$; Naïve n = 9, Control n = 9, Ox n = 9]. (C) Serum immunoglobulin E levels were measured in PD40 mice [one-way ANOVA: $F(2,15) = 28.92$, $P < 0.01$; Naïve n = 6, Control n = 6, Ox n = 6]. (D) The expression of a microglial inhibitory marker, *CD200R1*, was analyzed by quantitative reverse transcription polymerase chain reaction of the hypothalamus [one-way ANOVA: $F(2,9) = 0.42$, $P = 0.67$], hippocampus [one-way ANOVA: $F(2,9) = 0.71$, $P = 0.52$], prefrontal cortex [one-way ANOVA: $F(2,9) = 3.75$, $P = 0.06$], and amygdala [one-way ANOVA: $F(2,9) = 16.31$, $P < 0.01$] of all group mice, normalized to glyceraldehyde 3-phosphate dehydrogenase expression (Naïve n = 4, Control n = 4, Ox n = 4). Values are expressed as mean ± standard error of the mean ($^{*}p < 0.05$, $^{**}p < 0.01$).

chemokines, *monocyte chemotactic protein-1 (MCP-1)* and *Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES)*, which are known to be potent chemoattractants for monocytes and eosinophils, respectively, significantly increased in the Ox-treated ears (Fig. 2A). The expression of pro-inflammatory cytokines, *IL-1β* and *IL-6,* also increased significantly in the hyperplastic lesions of the Ox-treated mice (Fig. 2A). Furthermore, the Ox-treated mice showed a significant increase in the serum level of IgE on PD30 (Fig. 2B). These results indicated that multiple Ox challenges induced chronic inflammation, followed by Th2 dominant induction in the lesion sites, and a

predisposition to allergic conditions.

3.3. Neither behavioral abnormalities nor an increase in pro-inflammatory cytokine expression, but rather the primed state within the brain, was observed in the Ox-treated mice on PD40

On PD40, ear swelling ameliorated remarkably in the Ox-treated mice, although it remained statistically different from that observed in the naïve and control mice (Fig. 3A). Serum corticosterone level in the Ox-treated mice was normalized to the control level (Fig. 3B), whereas serum IgE levels in the Ox-treated mice were still higher than those in the control mice (Fig. 3C).

To examine the effects of early life stress on Ox-induced dermatitis in adolescent PD40 male mice, we examined behavioral abnormalities and pro-inflammatory cytokine expressions in several brain regions where stress could induce neuroinflammation. Contrary to expectations, we observed no abnormalities in behavioral tests determined by conditions such as anxiety, depression, and social interaction (Supplementary Fig. 1). In accordance with the results of behavioral tests, gene expression analysis also did not show any significant differences between the Ox-treated and control mice (Supplementary Fig. 2). Next, we examined whether early life stress from Ox-induced dermatitis could prime the brain immune system. The expressions of microglial activation markers (*Iba-1*, *CD11b*, *TLR4*), microglial inhibitory marker (*CX3CR1*), and astrocyte activation marker (*GFAP*) did not show any difference between the control and Ox-treated mice (Supplementary Fig. 3). However, the expression of microglial inhibitory marker, *CD200R1*, was more significantly downregulated in the amygdala of the Ox-treated mice compared to the control mice (Fig. 3D). The results indicated that microglia might settle as the primed state in the brain of the Ox-treated mice on PD40.

3.4. Ox-treated mice were more susceptible to systemic inflammation with LPS challenge at 4 h

To confirm the primed state in the brain of the Ox-treated mice, we assessed the expression of pro-inflammatory cytokines in the hypothalamus, hippocampus, PFC, and amygdala 4 h after administration of LPS (1 mg/kg) or saline intraperitoneally (Fig. 4A). As expected, the expression of *IL-6* in the hippocampus was significantly higher in the Ox-treated mice than that in the control mice 4 h after LPS administration (Fig. 4B, Supplementary Fig. 4), whereas the locomotor activity of the Ox-treated mice was similar to that of the control mice 4 h after LPS challenge (Supplementary Fig. 5). Furthermore, the number of Iba-1 (a microglial activation marker)-positive cells was significantly increased in the hippocampus and amygdala of the Ox-treated mice 4 h after LPS administration (Fig. 4C, 4D, and 4E).

3.5. Depressive-like behavior was apparent 24 h after LPS challenge in the Ox-treated mice

To investigate whether the susceptibility to systemic inflammation in the Ox-treated mice would affect their behavior, we assessed depressive-like and anxiety-like behaviors 24 h after LPS administration (Fig. 4 A). The total distance was more normalized in LPS-administered control mice compared to saline-administered control mice, whereas the Ox-treated mice administered with LPS did not fully recover from the decrease in locomotor activity (Fig. 5A). Furthermore, the Ox-treated mice apparently showed depressive-like behaviors 24 h after LPS administration in both the sucrose preference test and tail suspension test (Fig. 5B, C), whereas anxiety-like behavior was not more significantly observed in these mice compared to the control mice (Fig. 5A). Regarding pro-inflammatory cytokine expressions in brain regions 26 h after LPS administration, the expression of *IL-1β* was still upregulated in LPS-administered mice in all groups compared to that of the saline-administered mice, whereas the expression level of *IL-6* was normalized in LPS-administered mice of all groups (Supplementary Fig. 6).

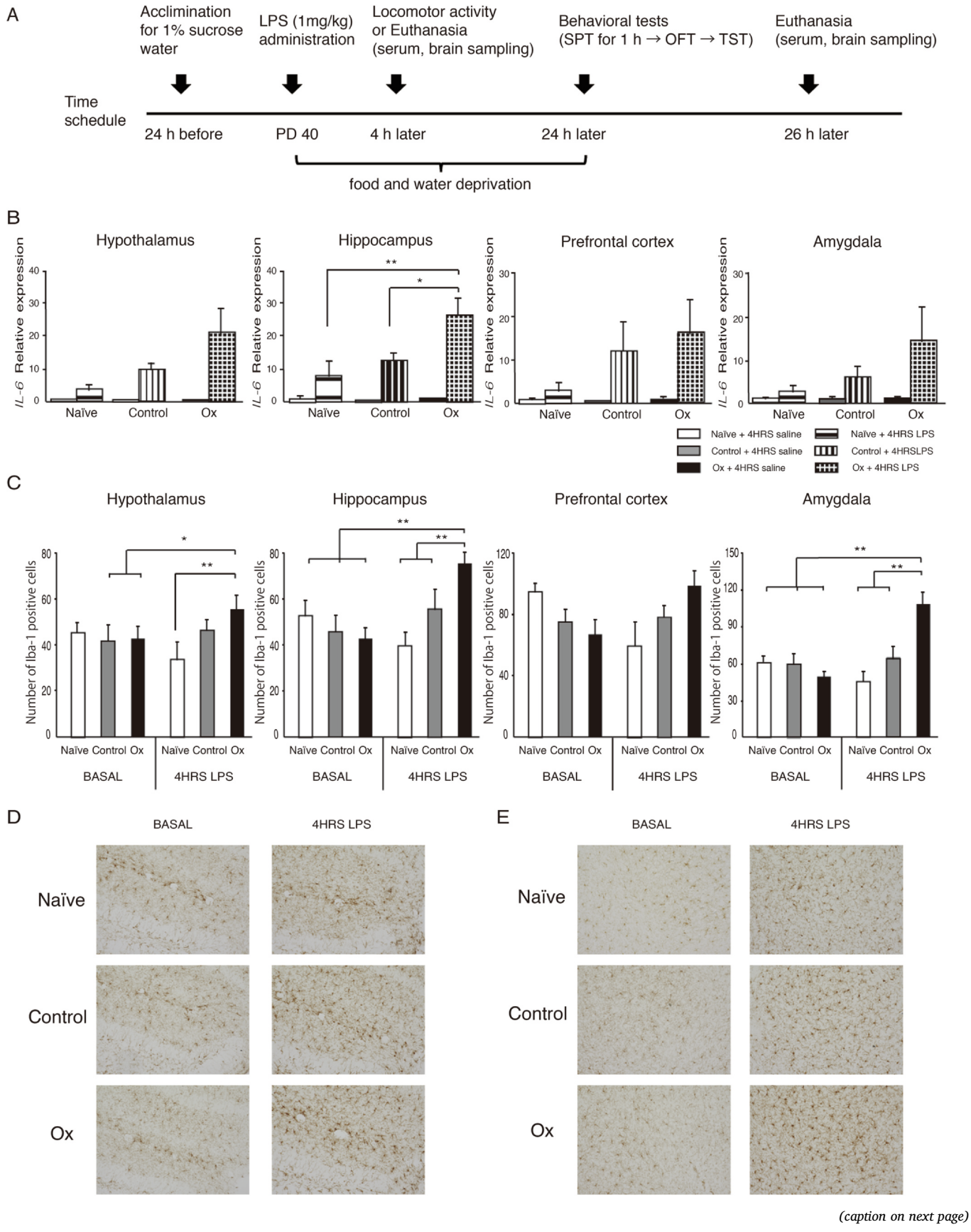
3.6. Expressions of indoleamine 2,3-dioxygenase and kynurenine 3-monooxygenase in the hippocampus, prefrontal cortex, and amygdala were exaggerated after LPS administration in the Ox-treated mice

The kynurenine metabolism pathway has recently been reported as a key pathway in inflammation-induced depression (O'Connor et al., 2009; Dantzer, 2017). In reference to the reports, we examined the expression of *indoleamine 2,3-dioxygenase (IDO)* and *kynurenine 3-monooxygenase (KMO)* in LPS-administered mice in each group. Interestingly, 4 h after LPS administration, the expression of *IDO* was upregulated in the hippocampus and PFC (Fig. 6). Surprisingly, the expression of *KMO* was remarkably increased in the amygdala of the Ox-treated mice 4 h after LPS administration (Fig. 6), whereas the enhancement of these enzyme expressions was normalized to control levels 26 h after LPS administration (Supplementary Fig. 7).

4. Discussion

AD is currently considered the earliest manifestation of atopic march, affecting approximately 20%–30% of infants, and some clinical trials are underway to prevent the progression of atopic march with proper treatment of AD during early life stage (Lowe et al., 2018). One of the main symptoms of AD is intense pruritus, and AD patients experiencing the symptom show a stress condition with an increase in salivary cortisol levels (Mizawa et al., 2013). Furthermore, AD not only predisposes patients to other allergic diseases but is also associated with psychiatric disorders, including depression, anxiety, ADHD, and autism (Silverberg et al., 2019). In the present study, we have provided the first report about the effects of allergic dermatitis in the early life period on adolescents' mental health. Our results suggest that chronic stressful events of allergic dermatitis during early childhood might cause microglia to be a priming state with high susceptibility to systemic inflammation within the brain, accompanied by an increased pro-inflammatory cytokine expression and an enhanced proliferation ability of microglia (Fig. 4B, 4C). Moreover, after an exaggerated response to systemic inflammation, depressive-like behaviors, as determined by sucrose preference and tail suspension tests, are apparent in the allergic dermatitis-induced animals (Fig. 5B and 5C). It is noteworthy that the enhancement of the expression of *IDO* and *KMO*, key enzymes of the kynurenine pathway (KP), was induced in the hippocampus, PFC, and amygdala after exposure to systemic inflammation (Fig. 6). The results indicated that the neuroinflammatory priming state induced by juvenile stress from allergic dermatitis generated the increased response for the neuroinflammation and then overproduction of pro-inflammatory cytokines caused abnormal tryptophan metabolism, which could exert behavioral abnormalities in a subject (Dantzer, 2017).

There are several reports that early life adverse events, such as maltreatment, parental neglect, and childhood abuse, are strongly associated with neuropsychiatric disorders, including depression and anxiety, in both humans and rodents (He et al., 2020; Hughes et al., 2017; Molet et al., 2014; Liu et al., 2017). Since these vulnerable periods during the development of the nervous system are sensitive to environmental insults, external influences during early life may, in turn, have profound effects on neuronal development processes (Goff and Tottenham, 2015; Rice and Barone, 2000). Researchers have long focused on the hypothalamic-pituitaryadrenal axis, limbic systems, and PFC, where early life stresses appear to primarily affect neurobehavioral development (Herman et al., 2005; Lai and Huang, 2011). Chronic pruritus, one of the main stressful events in AD, may also change the connectivity within the limbic systems in AD patients (Mochizuki et al., 2017). One group recently reported that male adult mice with AD-like lesions in the cheek, induced by repeated application of vitamin D3 analog, showed anxiety- and depressive-like behaviors,



(caption on next page)

Fig. 4. Augmented susceptibility to neuroinflammatory responses for lipopolysaccharide stimulation in oxazolone-treated mice. (A) Representation of the time schedule for LPS administration tests. All tests, except the final sacrifice, were carried out in all mice according to the time schedule. (B) The expression of pro-inflammatory cytokine, *IL-6*, was analyzed by quantitative reverse transcription polymerase chain reaction of the hypothalamus (*IL-6* [two-way ANOVA: model effect $F(2,27) = 3.02$, $P = 0.07$; administration effect $F(1,27) = 7.99$, $P < 0.01$; interaction effect $F(2,27) = 1.47$, $P = 0.25$]), hippocampus (*IL-6* [two-way ANOVA: model effect $F(2,27) = 6.62$, $P < 0.01$; administration effect $F(1,27) = 24.85$, $P < 0.01$; interaction effect $F(2,27) = 3.20$, $P = 0.06$]), prefrontal cortex (*IL-6* [two-way ANOVA: model effect $F(2,27) = 1.36$, $P = 0.27$; administration effect $F(1,27) = 2.57$, $P = 0.12$; interaction effect $F(2,27) = 0.68$, $P = 0.52$]), and amygdala (*IL-6* [two-way ANOVA: model effect $F(2,27) = 1.39$, $P = 0.27$; administration effect $F(1,27) = 4.78$, $P < 0.05$; interaction effect $F(2,27) = 0.72$, $P = 0.49$]) 4 h after lipopolysaccharide (LPS) (1 mg/kg) or saline administration, normalized to glyceraldehyde 3-phosphate dehydrogenase expression (saline groups; Naïve n = 4, Control n = 4, Ox n = 4, LPS groups; Naïve n = 6, Control n = 7, Ox n = 8). (C) Mean number of Iba-1-positive microglia was calculated before and 4 h after LPS administration in the hypothalamus [two-way ANOVA: model effect $F(2,48) = 1.35$, $P = 0.27$; administration effect $F(1,48) = 0.21$, $P = 0.65$; interaction effect $F(2,48) = 2.57$, $P = 0.09$], hippocampus [two-way ANOVA: model effect $F(2,48) = 1.73$, $P = 0.19$; administration effect $F(1,48) = 2.95$, $P = 0.09$; interaction effect $F(2,48) = 5.40$, $P < 0.01$], prefrontal cortex [two-way ANOVA: model effect $F(2,48) = 0.30$, $P = 0.74$; administration effect $F(1,48) = 0.06$, $P = 0.81$; interaction effect $F(2,48) = 6.41$, $P < 0.01$], and amygdala [two-way ANOVA: model effect $F(2,48) = 5.34$, $P < 0.01$; administration effect $F(1,48) = 5.86$, $P < 0.05$; interaction effect $F(2,48) = 11.57$, $P < 0.01$] (saline groups; Naïve n = 3, Control n = 3, Ox n = 3, LPS groups; Naïve n = 3, Control n = 3, Ox n = 3). Values are expressed as mean ± standard error of the mean ($^{*}p < 0.05$, $^{**}p < 0.01$). The representation of immunohistochemistry analysis for ionized calcium-binding adaptor molecule-1 (Iba-1) positive cells was shown in hippocampus (D) and amygdala (E) before and 4 h after LPS administration (scale bar, 25 μm). Original magnification: 200×.

along with neuroplastic changes in the reward circuitry, which is a specific limbic circuit (Yeom et al., 2020). Contrary to the report, our dermatitis model did not show any behavioral abnormalities without an additional insult. This could be mainly because of different durations of allergen-treatment and differences in the age of mice used in each experiment.

Neuroinflammation is one of the pathophysiological features of stress conditions, evoking a state of psychiatric disorders (Kim and Won, 2017). Microglia is a key player in neuroinflammation, a resident innate immune cell in the brain that can influence neuronal plasticity through the secretion of cytokines and the shaping of neuronal networks (Delpech et al., 2015). Activated microglia in a stress condition start to produce pro-inflammatory cytokines, IL-1β, IL-6, and TNFα, within the brain, specifically the hypothalamus, hippocampus, and PFC region (Johnson et al., 2019). In our experiment, PD40 adolescent male mice did not show any neuroinflammation signatures in the

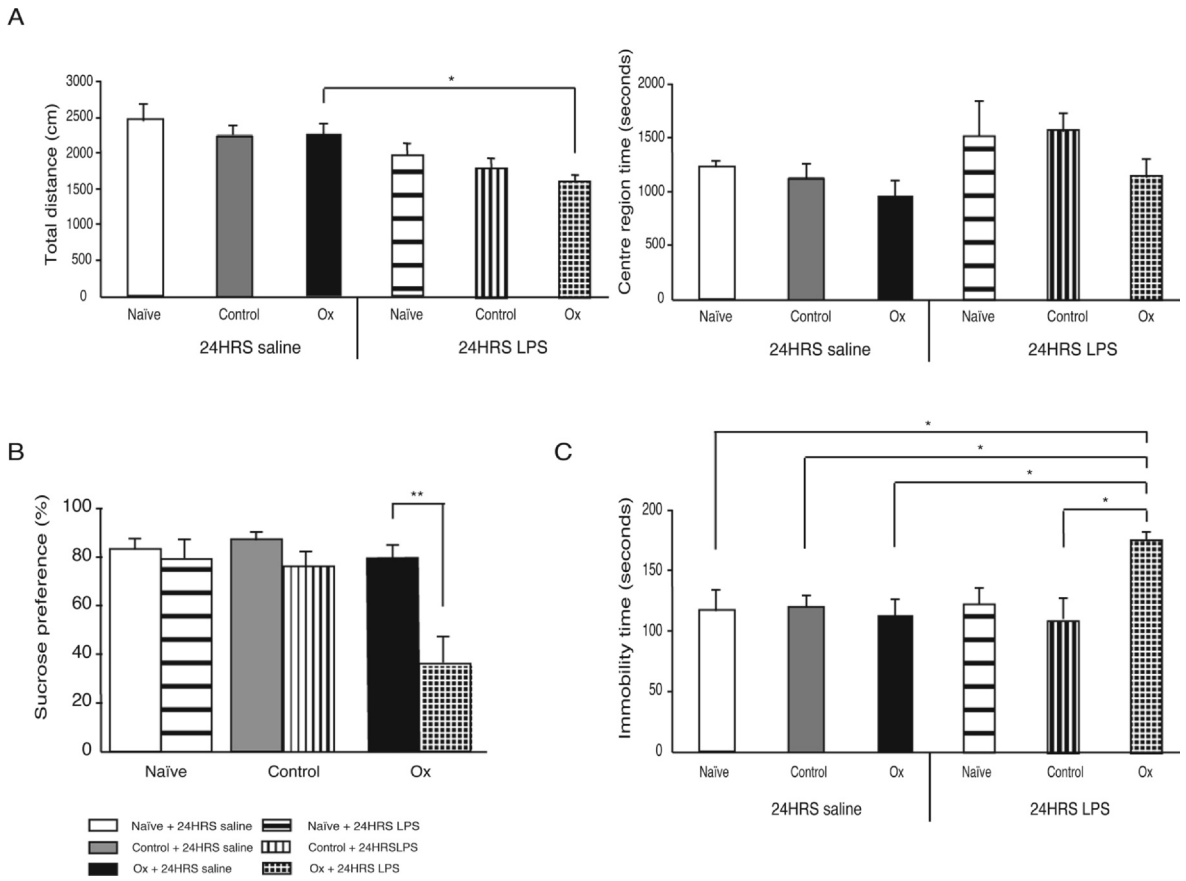


Fig. 5. Induction of depressive behavior 24 h after lipopolysaccharide (LPS) administration in oxazolone-treated mice.(A) The spontaneous locomotor activity (left panel [two-way ANOVA: model effect $F(2,33) = 2.34$, $P = 0.11$; administration effect $F(1,33) = 18.68$, $P < 0.01$; interaction effect $F(2,33) = 0.21$, $P = 0.81$]) and the time spent in the center region (right panel [two-way ANOVA: model effect $F(2,33) = 1.81$, $P = 0.18$; administration effect $F(1,33) = 4.13$, $P = 0.05$; interaction effect $F(2,33) = 0.30$, $P = 0.75$]) were measured in the open-field test (saline groups; n = 6, LPS groups; n = 6–8). (B) Sucrose preference was calculated in the sucrose preference test [two-way ANOVA: model effect $F(2,33) = 17.43$, $P < 0.01$; administration effect $F(1,33) = 23.80$, $P < 0.01$; interaction effect $F(2,33) = 8.29$, $P < 0.01$]. (C) Immobility time was counted in the tail suspension test [two-way ANOVA: model effect $F(2,33) = 3.97$, $P < 0.05$; administration effect $F(1,33) = 3.28$, $P = 0.08$; interaction effect $F(2,33) = 4.09$, $P < 0.05$] (saline groups; Naïve n = 6, Control n = 6, Ox n = 6, LPS groups; Naïve n = 6, Control n = 7, Ox n = 8). Values are expressed as mean ± standard error of the mean ($^{*}p < 0.05$, $^{**}p < 0.01$).

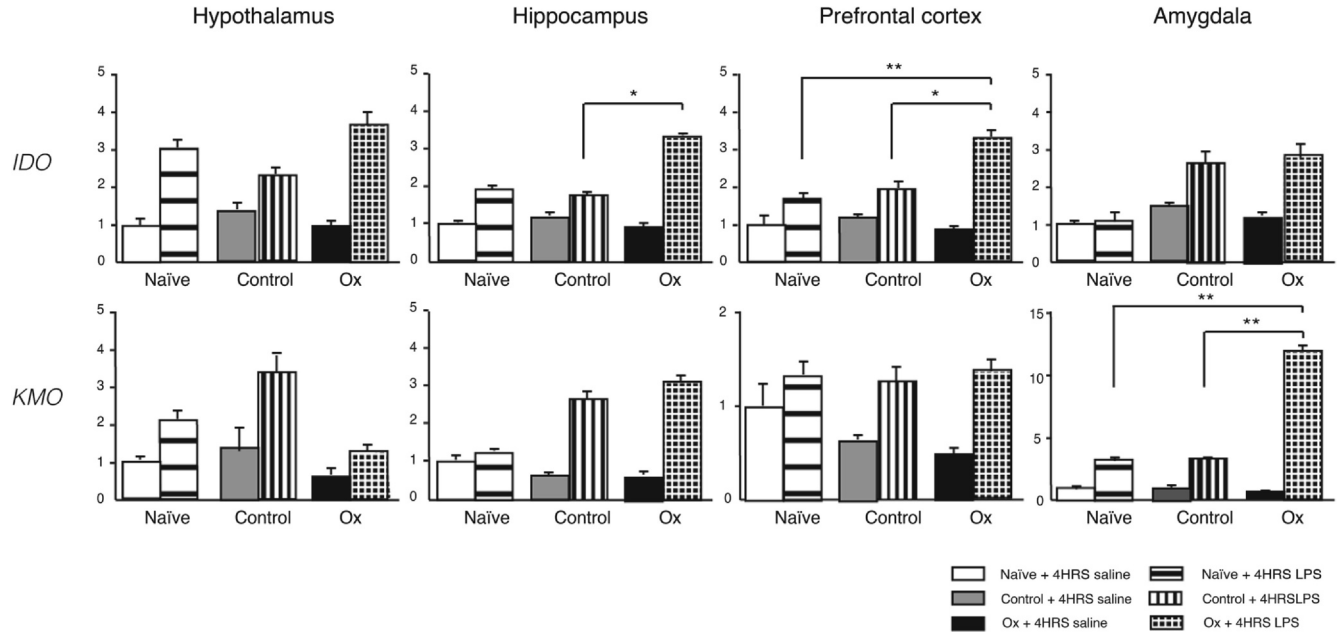


Fig. 6. Increased expression of kynurenine metabolic key enzymes, indoleamine 2,3-dioxygenase (*IDO*) and kynurenine 3-monooxygenase (*KMO*), in oxazolone-treated mice 4 h after lipopolysaccharide (LPS) administration. The expressions of *IDO* and *KMO* 4 h after LPS administration were analyzed by quantitative reverse transcription polymerase chain reaction of the hypothalamus (*IDO* [two-way ANOVA: model effect $F(2,18) = 0.56$, $P = 0.58$; administration effect $F(1,18) = 29.76$, $P < 0.01$; interaction effect $F(2,18) = 1.91$, $P = 0.18$], *KMO* [two-way ANOVA: model effect $F(2,18) = 3.48$, $P = 0.05$; administration effect $F(1,18) = 8.58$, $P < 0.01$; interaction effect $F(2,18) = 0.87$, $P = 0.43$]), hippocampus (*IDO* [two-way ANOVA: model effect $F(2,18) = 3.15$, $P = 0.07$; administration effect $F(1,18) = 28.46$, $P < 0.01$; interaction effect $F(2,18) = 4.16$, $P < 0.05$], *KMO* [two-way ANOVA: model effect $F(2,18) = 1.91$, $P = 0.18$; administration effect $F(1,18) = 30.14$, $P < 0.01$; interaction effect $F(2,18) = 5.25$, $P < 0.05$]), prefrontal cortex (*IDO* [two-way ANOVA: model effect $F(2,18) = 3.78$, $P < 0.05$; administration effect $F(1,18) = 33.28$, $P < 0.01$; interaction effect $F(2,18) = 7.07$, $P < 0.01$], *KMO* [two-way ANOVA: model effect $F(2,18) = 0.63$, $P = 0.54$; administration effect $F(1,18) = 9.35$, $P < 0.01$; interaction effect $F(2,18) = 0.63$, $P = 0.54$]), and amygdala (*IDO* [two-way ANOVA: model effect $F(2,18) = 3.15$, $P = 0.07$; administration effect $F(1,18) = 28.46$, $P < 0.01$; interaction effect $F(2,18) = 4.16$, $P < 0.05$], *KMO* [two-way ANOVA: model effect $F(2,18) = 11.3$, $P < 0.01$; administration effect $F(1,18) = 26.19$, $P < 0.01$; interaction effect $F(2,18) = 8.19$, $P < 0.01$]) normalized to glyceraldehyde 3-phosphate dehydrogenase expression (saline groups; Naïve n = 4, Control n = 4, Ox n = 4, LPS groups; Naïve n = 4, Control n = 4, Ox n = 4). Values are expressed as mean ± standard error of the mean ($^{*}p < 0.05$, $^{**}p < 0.01$).

hypothalamus, hippocampus, PFC, and amygdala after the induction of allergic dermatitis (Supplementary Fig. 2). This may be because the stress condition on PD40 subjects would be ameliorated with normalization of the serum corticosterone level (Fig. 3B), whereas the stress condition on PD30 subjects clearly exhibited an increase in the serum corticosterone level and PD30 Ox-treated mice showed suppressive responses for neuroinflammation within the brain (Fig. 1E, Supplementary Fig. 8).

Microglia comprise three main types, resting microglia, M1 microglia, and M2 microglia, and they can transform each other depending on their circumstances (Zhang et al., 2018). Interestingly, microglia cannot only adapt to their circumstances but also memorize an initial activation, called the priming state, leading to an exaggerated response to a second stimulation (Calcia et al., 2016). The priming state of microglia was first demonstrated in prion model mice (Cunningham et al., 2005), while subsequent studies revealed that several chronic stimuli could evoke microglia priming, including stress, aging, traumatic CNS injury, and neurodegenerative disease (Norden et al., 2015; Niraula et al., 2017). Although the precise mechanism of priming state in the microglia has not been elucidated, some reports suggest that pre-exposure to glucocorticoids and activation of the sympathetic nervous system could sensitize the microglia to neuroinflammation (Frank et al., 2014; Liu et al., 2018; Sapolsky, 2015; Fonken et al., 2018). CD200R1 is a key regulator of neuroinflammation, exclusively expressed on the microglia and other myeloid cells in the brain, and the downregulation of CD200R1 leads to the neuroinflammatory priming state under stress conditions (Frank et al., 2018). One report showed that corticosterone directory downregulates the expression of *CD200R1* in *ex vivo* microglia (Fonken et al., 2016). In addition, increased microglial proliferation is also involved in neuroinflammatory priming under stressful conditions (Fonken et al., 2018; Lehmann et al., 2016). It is known that microglia are morphologically and transcriptionally heterogeneous and maintain their population with rapid turnover through proliferation and apoptosis (Hammond et al., 2019; Askew et al., 2017). An excellent work using *in vivo* imaging was recently reported that microglial self-renewal was slow and stochastic under basal condition, while microglia very rapidly self-renew locally in response to injury (Monique, 2020). Furthermore, priming microglia caused by early life alcohol exposure showed the prompt increase of Iba-1-positive microglia 2 h after LPS challenge (Chastain et al., 2019). In agreement with these reports, our allergic dermatitis model also showed the downregulation of *CD200R1* expression in the amygdala under basal conditions in PD40 (Fig. 3D), and the enhanced proliferation of activated microglia in the hippocampus and amygdala 4 h after LPS administration (Fig. 4C); however, precise *ex vivo* examination and morphological analysis are warranted in the future.

Early life stress has been implicated in the development of stress-related psychiatric disorders across the patient's life (VanTieghem and Tottenham, 2018). As early life is a critical period of vulnerability for the developing nervous system, stressful events in this period result in behavioral and physiological alterations in later life (Rice and Barone, 2000). A critical period exists in the immune system, as well as in the nervous system, and these systems are inextricably linked during early life development (Danese and Lewis, 2017). Since early life stress influences the long-term functional changes in microglia through epigenetic alterations, the effect of microglial priming caused by the stress would last across the lifetime of the person (Johnson and Kaffman, 2018; Chastain et al., 2019). It should be noted that the recruitment of

other immune cells into the brain, including monocytes and lymphocytes, also contributes to neuroinflammatory priming (Xu et al., 2010; Ritzel et al., 2016; McKim et al., 2018). Some reports suggested that Th1 lymphocytes contributed to neurotoxic functions and Th2 lymphocytes served a neuroprotective modulation purpose via microglial regulation (Ta et al., 2019; Quarta et al., 2020). Conversely, our allergic dermatitis model, whose primed microglia settled in the brain, showed Th2-dominant immunity (Fig. 2A). It was also reported that the Th2-produced cytokines IL-4 and IL13 could lead to developmental disorders through the regulation of microglial polarization in another allergic condition (Kalkman and Feuerbach, 2017). In our allergic dermatitis model, peripheral immune reactions for LPS administration tended to be different from neuroinflammatory reactions within the brain (Supplementary Fig. 9). Further investigations are necessary to elucidate how the other immune cells interact with microglia under conditions of early life stress, leading to the neuroinflammatory priming.

A number of studies have suggested that an imbalance of tryptophan metabolism along the KP would influence depressive behavior in a systemic inflammatory condition (Campbell et al., 2014; Schwarcz et al., 2012). IDO and KMO are key limiting enzymes of KP in the inflammation-induced imbalance of tryptophan metabolism. Abnormal regulation of IDO or KMO results in depressive behaviors in patients with major depressive disorder and LPS-challenged animals (Savitz et al., 2015; O'Connor et al., 2009; Parrott et al., 2016a). INF-γ signaling is the canonical pathway that induces *IDO* expression through STAT1 or NF-κB activation, whereas pro-inflammatory cytokines, IL-1β, IL-6, and TNFα, could induce *IDO* and *KMO* expression through an alternative pathway, the INF-γ-independent pathway (Konan and Taylor, 1996; Zunszain et al., 2012; Wang et al., 2010). In this study, the regions of high response to additional systemic inflammation were different within the brain for pro-inflammatory cytokines, regulators of microglial activation, and KP key enzymes. This might be because the activation of microglia depends on the type of stimulations in a region-specific manner, and the response to LPS administration is also regulated spatially and temporally within the brain (André et al., 2008; Spencer et al., 2019; Parrott et al., 2016b). Based on these reports, our results suggest that allergic-dermatitis conditions in early life would provoke depressive-like behaviors in adolescent life with increased *IDO* and *KMO* expression within the brain, which might be induced by the overproduction of pro-inflammatory cytokines in response to systemic inflammation.

This study has some limitations. First, the lesions in our allergic dermatitis model are limited to the ears because stress from handling should be minimal, while mental health comorbidities in AD patients depend on the severity of dermatitis, which is clinically defined by the area of dermatitis lesions (Silverberg et al., 2019). Second, the duration of stress notably affects mental health problems involving microglial plasticity. In our pilot study using adult mice, a dermatitis lesion with epidermal hypertrophy appeared 14 days after sensitization with initial treatment (data not shown), and we confirmed the increase in serum stress hormone, corticosterone, 30 days after sensitization (Fig. 1E). Considering these results, the stress condition of our allergic dermatitis model was regarded as chronic stress, where a broad range of the brain would be affected by changes in microglial structure and function (Walkera et al., 2013). Third, the water and food deprivation might affect motivated behavior. Recently, energy-regulating neuroendocrine hormone, leptin, has been reported as a regulator of dopaminergic system (Cordeiro et al., 2019). Since we would like to measure sucrose water consumption without variation bias of the last meal timing and examine the effect of systemic inflammation just after recovery from sickness behavior, we performed behavioral tests after 24 h food and water deprivation in parallel with LPS administration (Fig. 4A). Finally, we only examined male mice in this report because of the effect of sex hormones on the central nervous system. Since not only the incidence of stress-related disorders, such as depression and anxiety, but also the regulation of neuroinflammatory priming to stress have a sex difference in both human and animal models (Donner and Lowry, 2013; Rincón-Cortés et al., 2019), further investigations of sex differences in the effect of early life stress with allergic dermatitis on mental health in adolescent life are necessary.

In conclusion, this is the first report to reveal that early life stress from allergic dermatitis affects mental health in adolescent life. Early life stress from allergic dermatitis could cause a priming state for second stressful events. This results in adolescent animals being highly susceptible to systemic inflammation, leading to depressive behaviors with an abnormal kynurenine metabolic pathway. These findings provide further evidence that early life stress from allergic dermatitis could cause mental health problems in adolescence and shed more light on potential new therapeutic strategies for mental health comorbidities in AD patients.

Author contributions

OH conceived and performed the experiments; HK and YN helped to perform behavioral tests; MY gave a helpful advice; MS and KW supervised the work; OH and MS wrote the manuscript. All authors approved the final submission and publication of this manuscript.

Acknowledgments

We thank Ms. Yasuko Nakamura and Ms. Hiromi Fujita for their laboratory assistance and animal care. This work was supported by the following grants: KAKENHI grant number 15K06730 (to MS) and 19K17349 (to OH), and Intramural Research Grant for Neurological and Psychiatric Disorders funded by the National Center of Neurology and Psychiatry (NCNP), Japan (to MS).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2020.09.013.

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