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Research paper

Effects of adolescent alcohol exposure on oligodendrocyte lineage cells and myelination in mice: Age and subregion differences

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ABSTRACT

Adolescence is an important phase for the structural and functional development of the brain. The immaturity of adolescent brain development is associated with high susceptibility to exogenous disturbances, including alcohol. In this study, the acquisition of conditioned place preference (CPP) in adolescent mice by alcohol (2 g/kg) and the parvalbumin-positive interneurons (PV^+ interneurons), oligodendrocyte lineage cells (OPCs), and myelination in the medial prefrontal cortex (mPFC) were assessed. We aim to determine the age- and subregional-specificity of the effects of alcohol. Alcohol (2 g/kg) was injected intraperitoneally on even days, and saline was injected intraperitoneally on odd days. The control group received a continuous intraperitoneal injection with saline. Differences in alcohol-induced CPP acquisition were assessed, followed by immunohistochemical staining. The results showed a pronounced CPP acquisition in 4- and 5-week-old mice. In the mPFC, there were reduced PV^+ interneurons and OPCs in 3-week-old mice and reduced oligodendrocyte numbers in 4-week-old mice. The 5-week-old mice showed impaired myelination and a decrease in the number of PV^+ interneurons, mature oligodendrocytes, and OPCs in the mPFC. Since the alterations in 5-week-old mice are more pronounced, we further explored the mPFC-associated subregional-specificity. In the alcohol-exposed mice, the oligodendrocyte numbers were decreased in the anterior cingulate cortex (ACC), PV^+ interneuron numbers were declined in the prelimbic cortex (PL), and the number of oligodendrocytes, PV^+ interneurons, and OPCs was also decreased with impaired myelination in the infralimbic cortex (IL). Our data suggest that adolescent alcohol exposure notably affected the acquisition of CPP, myelin formation, and the counts of PV^+ interneurons, mature oligodendrocytes, and OPCs in the mPFC in 5-week-old mice. Also, the IL subregion was the worst-affected subregion of the mPFC in alcohol-exposed 5-week-old mice. It reveals that the effects of alcohol on adolescence and its mPFC myelination show obvious age- and subregional-specificity.

1. Introduction

Alcohol dependence, also known as alcohol use disorder, is one of the significant health issues facing society. It is defined as the collection of impaired brain function and uncontrolled behavior, including disorders represented by compulsive heavy drinking and a loss of control over alcohol intake (Carvalho et al., 2019; Luderer et al., 2021; Nutt et al., 2019). With social and economic development, the increase in the number of adolescent drinkers and the proportion of severe alcoholics (Siqueira & Smith, 2015; The Lancet Public, 2018) has caused widespread social burden (economic burden, social security problems) and health consequences (such as traffic accidents, suicide, social violence, and liver disease) and is associated with increasing mortality (Knox et al., 2019; Kraus et al., 2019; Rehm et al., 2013). Moreover, alcohol is more likely to interrupt key processes of brain development during adolescence, a critical period of development, potentially resulting in cognitive dysfunction and an increased risk of chronic alcohol dependence (Guerri & Pascual, 2010; Patrick et al., 2013). Alcohol

Abbreviations: ACC, anterior cingulate cortex; ANOVA, One-way analysis of variance; AOD, average optical density; AUD, alcohol use disorder; CNPase, 2', 3'-cyclic'adenylate-3'-phosphodiesterase; CPP, conditioned place preference; DAB, diaminobenzidine; IL, infralimbic cortex; MBP, myelin basic protein; MPFC, medial prefrontal cortex; NG2, neuron-glial antigen 2; OL, mature oligodendrocyte; OPCs, oligodendrocyte precursor cells; P1, postnatal day 1; PDGFRα, platelet-derived growth factor receptor α; PL, prelimbic cortex; PV^+ interneurons, parvalbumin-positive interneurons.

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dependence is a brain disease involving the brain reward circuit, and patients with alcohol dependence often face a significantly increased risk of anxiety, depression, and cognitive impairment (Gilpin & Koob, 2008).

The medial prefrontal cortex (mPFC) integrates information from many cortical and subcortical regions and projects the integrated information to the subcortical regions that control emotion, motivation, and impulsivity. It plays a crucial role in higher executive, cognitive processes, emotion regulation, motivation, and social competence (Goldstein & Volkow, 2011; Miller & Cohen, 2001). Studies have proved that excessive alcohol consumption alters fear memory extinction and mPFC neuroplasticity expression in adolescent mice (Lawson et al., 2022), brain metabolism, and functional connectivity in the prefrontal cortex (Tang et al., 2019). Simultaneously, in male rats with chronic alcohol exposure, significant dysfunction of the medial prefrontal cortical-basolateral amygdala circuit is observed (Crofton et al., 2022). Alcohol-dependent individuals, who usually have a long-term history of alcohol use, show enhanced alcohol seeking, cognitive dysfunction and impaired executive function in the mPFC (Goldstein & Volkow, 2011).

Parvalbumin-positive interneurons (PV^+ interneurons) belong to GABAergic interneurons, essential for normal brain function (Hu et al., 2014). Myelin is the structural basis of signal transduction between neurons, and the destruction of myelin seriously affects brain function, which relies heavily on the synchronization of nerve impulses (Lee et al., 2012). Myelin formed by oligodendrocytes in the central nervous system, acts as insulating shells and provides nutritional and metabolic support to neural axons (Dai et al., 2003; Du & Dreyfus, 2002). Studies have shown that alcohol exposure decreases the count of PV^+ interneurons in mPFC (Rice et al., 2019), thereby affecting its role in keeping the balance of neuronal excitation/inhibition and modulating the reward-seeking process (Nahar et al., 2021). Rodent-related studies have demonstrated that alcohol exposure impacts the expression of markers at multiple phases of oligodendrocytes, including oligodendrocyte precursor cells (OPCs), pre-myelinated oligodendrocytes, and mature myelinated oligodendrocytes (Niedzwiedz-Massey et al., 2021). Chronic intermittent alcohol vapor exposure leads to alcohol dependence, modifies pyramidal neuron activity, decreases mPFC glial progenitors' number (Kim et al., 2015), and inhibits myelin basic protein (MBP) expression (Somkuwar et al., 2016; Vargas et al., 2014).

Postnatal days 21–35 in mice have been demonstrated to be critical for oligodendrocyte development and myelination in the mPFC (Makinodan et al., 2012), crucial for behavioral control and cognitive function in adulthood (Giedd, 2004; McDougall et al., 2018; Mengler et al., 2014). Excessive alcohol use during this period may interfere with normal neuronal development processes, potentially having long-term impacts on brain function and behavior. Simultaneously, the rodent mPFC could be divided into the anterior cingulate cortex (ACC), prelimbic cortex (PL), and infralimbic cortex (IL) in view of structural and connectivity features (Green & Bouton, 2021). Relevant investigations have revealed significant functional heterogeneity of the ACC, IL, and PL subregions in mPFC (Capuzzo & Floresco, 2020; Heidbreder & Groenewegen, 2003).

Therefore, this study compared the behavioral performance of adolescent mice aged 3, 4, and 5 weeks after alcohol exposure, the count of PV^+ interneurons, oligodendrocyte lineage cells, and myelination abnormalities in mPFC, to identify the age when alcohol causes a comparatively significant effect. Meanwhile, based on the age most significantly affected by alcohol exposure, we continued to explore the differences in the number of PV^+ interneurons, oligodendrocyte lineage cells, and myelination abnormalities following alcohol exposure in the ACC, PL, and IL subregions of the mPFC to identify the subregions most significantly affected by alcohol exposure.

2. Materials and methods

2.1. Animals

The C57BL/6 J background mice (fertile) were brought from the Jackson Laboratory in the United States and conserved and bred using the Research Platform of the Experimental Animal Center of the Army Medical University's Second Affiliated Hospital. The experimental wild-type (WT) mice were fed only basal feed and mated with one another to produce experimental WT offspring mice. In all animal studies, male mice were used. All mice were maintained in a pathogen-free, temperature- and humidity-stabilized environment with a 12-hour light/dark alternation and free access to food and water. The experimental design, experimental process and animal execution method were all in line with the requirements of the Ethical Review Committee for experimental animal welfare of the Army Medical University (AMUWEC202051).

2.2. Drugs and drug treatment

Basal feed was purchased from Chongqing Watson Biotechnology Co., Ltd. In this experiment, male mice of 3, 4, and 5-week-olds (P21, P28, and P35) from normal WT mice with a C57BL/6 background were divided into six groups, with six mice in each group: (i) 3WT+ SAL; (ii) 3WT+ ETOH; (iii) 4WT+ SAL; (iv) 4WT+ ETOH; (v) 5WT+ SAL; (vi) 5WT+ ETOH. The alcohol was diluted to 20 % (v/v) in 0.9 % saline, after which 20 % alcohol was administered intraperitoneally (i.p.) to mice at a dose of 2 g/kg during the treatment period of the conditioned place preference (CPP) experiment for 12 days (Martín-Sánchez et al., 2019).

2.3. CPP

The CPP apparatus consists of two equal-sized (15×15×15 cm) conditioning compartments: one compartment has a black wall and smooth black round hole floor made of 16-gauge stainless steel with many 6.4 mm round holes; the other has a white wall and a rough black mesh floor made of 2.3 mm stainless steel rods spaced 6.4 mm apart. There is a sliding door between the two compartments, which can be closed or opened to allow the free movement of experimental animals. The CPP protocol (Fig. 1) is based on references (Al Mansouri et al., 2014; Maiya et al., 2021), and includes three stages: habituation phase, conditioning phase, and testing phase, ensuring consistent environmental conditions such as light, tone, and odor in the compartment during the experiment. Briefly, each phase includes:

Habituation phase (Days 1–3): The mice were placed in the middle of the CPP apparatus and the sliding door was opened, allowing them to move freely for 15 min per day for 3 consecutive days. Saline was injected intraperitoneally daily to mitigate the effects of the experimental procedure on the mice. On Day 3, the time spent in the two compartments over a 15-minute period was recorded as baseline time and served as an indicator of the natural preference of the mice. The side with the longer residence time was defined as the preferred compartment, while the side with the shorter residence time was termed the non-preferred compartment. Simultaneously, mice that displayed a strong preference for either compartment (residence time in any compartment >70 %) were excluded from the experiment.

Conditioning phase (Days 4–11): Each mice was trained once a day. For the alcohol group, a 20 % alcohol solution was injected intraperitoneally on the 4th, 6th, 8th, and 10th days. The same volume of saline was injected intraperitoneally on the 3th, 5th, 7th, and 9th days. Five minutes after injection, mice were placed in either the non-preferred or preferred compartment for 15 min, respectively. Saline group: saline was injected throughout the experiment. The training time is fixed between 8 a.m. and 9 a.m. each day.

Testing phase (Days 12): Five minutes before the test, saline was injected intraperitoneally. The mice were placed in the middle of the

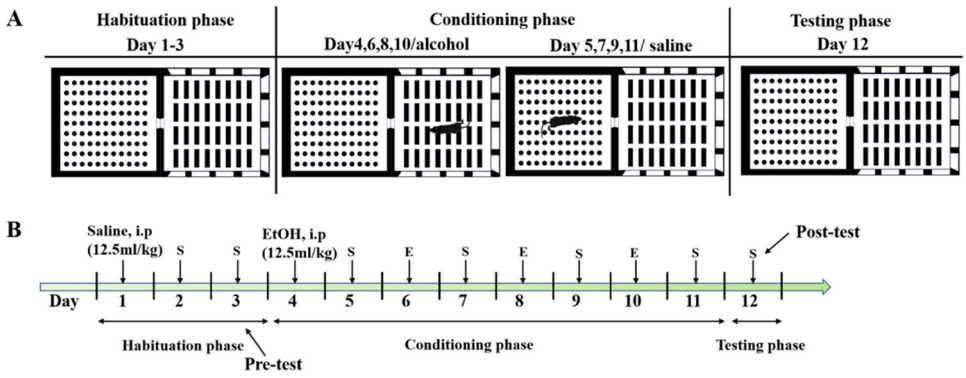

Fig. 1. CPP behavioral paradigm and testing schedule.

CPP apparatus, and the sliding door was opened to allow them to move freely. After recording the dwell time of the mice in the preferred or non-preferred compartment within 15 min, the CPP score was counted to reflect the acquisition of CPP in the mice. After each test, the instrument was thoroughly cleaned.

CPP scores were calculated as follows: Time difference (s) = time spent in the non-preferred compartment after the conditioning phase -time spent in the non-preferred compartment on day 3 of the habituation phase. The greater the time difference, the more obvious the performance of CPP in mice, suggesting that the reward effect of alcohol is more obvious.

2.4. Sample preparation

After the CPP test, mice were anesthetized with pentobarbital sodium (70 mg/kg). Their limbs were fixed, the heart was fully exposed, and saline was infused from the left ventricle and drained from the right atrial appendage. After the blood was emptied, fixation with 4 % paraformaldehyde was continued. After the limbs of the mice were stiff, the perfusion was ended, and the mice brain tissue containing the medial prefrontal cortex was removed and placed in 4 % paraformaldehyde and fixed for 24 h. The brain tissues were dehydrated in 10 %, 20 %, and 30 % gradient sucrose solution, respectively. After dehydration, the brain tissues were embedded in paraffin. Refer to Koss et al.'s study, which found no significant effects of adolescent alcohol exposure on brain weight and mPFC volume in adult mice (Koss et al., 2012). A series of coronal slices with a thickness of 3.5 μm were collected over the entire range of the mPFC (Bregma between + 1.98 and + 1.78 mm). The collected brain slices were placed in a slice box and stored at 25°C.

2.5. Immunohistochemical staining

The left or right hemisphere sections of mice were randomly selected and stained with universal kit (mouse/rabbit polymer method detection system) (PV-6000, ZSGB; China) and diaminobenzidine (DAB) chromogenic kit. The paraffin sections were heated at 56°C for 2 h, then deparaffinized and hydrated. The antigen was repaired by a microwave oven with low heat for 20 min, and then washed for 3 times in 0.1 M PBS for 3 min each time. Endogenous peroxidase blockers (reagent 1, PV-6000, ZSGB; China) were incubated for 10 min at 25°C and then washed three times with 0.1 M PBS for 3 min each time. Then, the sections were incubated with anti-CNPase antibody (1:500, Abcam, Catalog #ab6319), anti-PDGFRα antibody (1:500, Abcam, Catalog #ab203491), anti-MBP antibody (1:1200, CST, Catalog #78896 S), and anti-parvalbumin antibody (1:200, CST, Catalog #80561 S) overnight at 4°C. The next day, the incubated sections were removed, incubated for 1 h at 25°C, and washed 3 times in 0.1 M PBS for 3 min each time. An appropriate amount of conjugated goat anti-mouse/rabbit IgG polymer (reagent 2, PV-6000, ZSGB; China) was then incubated at 25°C for 25 min and washed 3 times in 0.1 M PBS for 3 min each time. Diaminobenzidine solution (DAB, ZLL-9018, ZSGB; China) staining occurred for about 5–8 min. Then, gradient alcohol (75 %, 75 %, 95 %, 95 %, 100 %, and 100 %; 10 s each) and xylene (2×10 s) were used for dehydration. Referring to Allen's Brain Atlas (Wang et al., 2020b), the boundaries of the mPFC as well as its ACC, PL, and IL subregions were identified as regions of interest (ROI). Finally, the sections were observed under an Olympus orthotopic fluorescence microscope to obtain representative images. ImageJ was used for experimental image analysis.

2.6. Immunofluorescence staining

Suitable brain tissue sections were selected, placed in 0.1 M PBST and PBS buffer, and washed 3 times for 5 min each. The sections were then placed in freezing repair solution for 30 min for antigen repair, followed by 3 washes in 0.1 M PBST and PBS buffer for 5 min each. Sections were treated with goat serum blocking solution for 1 h to reduce nonspecific binding. Following blocking, brain tissue sections were incubated in a primary antibody solution containing anti-NG2 (1:200, Sigma-Aldrich, Catalog #MAB5384-I) and anti-PDGFRα (1:500, Abcam, Catalog #ab203491) at 4°C overnight. The following day, the brain tissue sections were taken out and washed 3 times in 0.1 M PBST and PBS buffer for 5 min each. Incubate in Cy3-conjugated Affinipure Goat Anti-Mouse antibody (1:500, Proteintech, Catalog #SA00009–1) and Alexa Fluor 488® Goat Anti-Rabbit antibody (1:500, Invitrogen, Catalog #A-11008) secondary antibody solutions for 1 h and washed again to remove unbound secondary antibodies. Following staining with a DAPI antifade agent containing fluorescence, the slides were sealed with coverslips, and the sections were observed under a fluorescence microscope, with representative images obtained. ImageJ analysis software was used for subsequent analysis and processing of the images.

2.7. Image quantification

ImageJ software was used to count the number of cells in the ROI. Measure the ROI's area first. Load the experimental image and use the "Set Scale" option to set a fixed scale. The ROI was selected by referring to Allen's Brain Atlas (Wang et al., 2020b), and the area was measured by the "Measure" option under the "Analyze" menu. Next, cells in the ROI were manually counted. Load the experimental image and use the "Set Scale" option to set a fixed scale. Select the same ROI and use the "Cell Counter" plugin to count it. Finally, the manual counting results were divided by area to get the number of cells per unit area. The average optical density (AOD) of MBP was calculated using ImageJ software. Load the experimental images, set a fixed scale and ROI, and ensure that the boundaries of the ROI are consistent with the extent of the mPFC and its ACC, PL, and IL subregions. Convert the image to 8-bit grayscale and

set a threshold (Image→Adjust→threshold). Select the "Area" and "Integrated Density" options under "Measurements," and then choose the "Measure" option under the "Analyze" menu. Divide "Integrated Density" by "Area" to get the AOD value of the selected ROI. For all measurements, 6 mice were taken, and at least 3 sections per mice were analyzed.

2.8. Statistical analysis

All data were expressed as mean ± SEM and statistically evaluated using SPSS 20 software. One-way analysis of variance (One-way ANOVA) was used for data comparisons between multiple groups for a single variable; multivariate analysis of variance (multivariate ANOVA) was used for data comparisons between multiple groups involving multiple variables; and Student's t-tests were used for comparisons between two groups. $P < 0.05$ indicates a statistically significant difference.

3. Results

3.1. Alcohol-induced CPP in WT mice at different ages

CPP is one of the classical experimental models to evaluate the psychological dependence of drugs. It is also an effective tool widely utilized in numerous laboratories to evaluate the CPP of experimental animals and to discover effective drugs for addiction treatment. We tested the difference in CPP acquisition in 3, 4, and 5-week-old (P21, P28, and P35) WT mice after exposure to alcohol or saline. The retention time for 3-week-old WT mice in one compartment of the CPP apparatus after intraperitoneal injection of alcohol or saline was > 630 s, which did not comply with the inclusion criteria for the CPP experiment. Consequently, the CPP acquisition in 3-week-old mice after alcohol or saline treatment was not reflected in the chart. The baseline pre-test time spent in the non-preferred compartment was resemble for 4 groups of mice (Table 1, Fig. 2A, one-way ANOVA, $F(3,20) = 0.905$, $p = 0.456$). Compared with their respective pre-test time, the post-test time spent in the non-preferred compartment in the 4WT+ETOH group and the 5WT+ETOH group was significantly increased (Table 1, Fig. 2A, $t = -4.545$, $p < 0.01$; $t = -9.102$, $p < 0.01$).

Comparing the CPP scores of the mice in each group, a multivariate ANOVA suggested a significant main effect of treatment ($F_{treatment} = 85.470$, $p = 0.000$), while the age main effect and treatment × age interaction were not significant ($F_{age} = 1.547$, $p = 0.228$; $F_{treatment \times age} = 0.240$, $p = 0.630$). Moreover, as shown in Fig. 2B, CPP scores in the 4WT+ETOH group significantly increased by 47.0 % after alcohol exposure compared with the 4WT+SAL group ($t = -7.786$, $p < 0.01$); CPP scores in the 5WT+ETOH group significantly increased by 63.7 % after alcohol exposure compared with the 5WT+SAL group ($t = -5.886$, $p < 0.01$). This demonstrated that alcohol exposure caused significant CPP acquisition in 4 and 5-week-old WT mice.

Data was examined for normality, and all data were presented as mean ± SEM for time spent in seconds during the pre-test and post-test, $n = 6$. ** $p < 0.01$, compared with pre-test.

Table 1
Effect of alcohol exposure on time spent in non-preferred compartment.

Group	Pre-test (s)	Post-test (s)
4WT+SAL	336.2 ± 19.71	356.7 ± 17.63
4WT+ETOH	360.2 ± 28.02	529.7 ± 24.61**
5WT+SAL	331.8 ± 17.91	365.2 ± 13.32
5WT+ETOH	312.8 ± 13.41	511.8 ± 17.27**

3.2. Effects of alcohol exposure on parvalbumin-positive interneuron numbers in mPFC of WT mice at different ages

The medial prefrontal cortex seamlessly integrates information from various sources, facilitating the execution of complex functions, including behavioral control, working memory, and learning. It is paramount to overseeing cognitive processes, emotion regulation, motivation, and social ability. PV^+ interneurons are pivotal in maintaining the ideal excitation/inhibition balance through feedback and feedforward inhibition (Rice et al., 2019). A diminution in PV^+ interneurons is robustly linked with emotional and cognitive challenges in both mice and humans (Bissonette et al., 2015; Lodge et al., 2009). Therefore, this study proceeded to analyze the influence of alcohol exposure on the count of PV^+ interneurons in the mPFC of WT mice at ages 3, 4, and 5 weeks, given the prior noted variations in CPP acquisition post alcohol exposure in WT mice at disparate age weeks.

The immunohistochemical staining results are shown in Fig. 3. The multivariate ANOVA suggested significant main effects for both treatment and age ($F_{treatment} = 8.568$, $p = 0.004$; $F_{age} = 26.094$, $p = 0.000$), but no overall effect for the treatment × age interaction ($F_{treatment \times age} = 1.633$, $p = 0.200$). Compared with the 3WT+SAL group, the count of PV^+ interneurons in the mPFC of the 3WT+ETOH group decreased by 11.2 % after alcohol exposure ($t = 2.093$, $p < 0.05$). No salient difference in the count of PV^+ interneurons was observed between the 4WT+SAL group and the 4WT+ETOH group ($t = 0.347$, $p > 0.05$). Moreover, the PV^+ interneuron quantity in the mPFC of the 5WT+ETOH group was significantly diminished by 10.0 % compared to the 5WT+SAL group after alcohol exposure ($t = 2.555$, $p < 0.05$).

3.3. Effects of alcohol exposure on mature oligodendrocyte numbers and myelin in mPFC of WT mice at different ages

The maintenance of normal function in mPFC is closely related to mature oligodendrocytes and myelin. Studies have shown that mPFC exhibits varying degrees of myelin fragmentation and disorder under acute alcohol exposure (Newville et al., 2017; Newville et al., 2022). Myelin basic protein (MBP) and 2′, 3′-cyclic'adenylate-3′-phosphodiesterase (CNPase), regarded as expression markers of myelin and mature oligodendrocytes, can fully reflect the changes of myelin and mature oligodendrocytes. Therefore, we further investigated the count of mature oligodendrocytes ($CNPase^+$ cells) and the average optical density (AOD) of myelin in mPFC after alcohol exposure based on the observations in 3-, 4-, and 5-week-old WT mice.

First, the AOD values of myelin in mPFC were compared between groups of mice. A multivariate ANOVA suggested a significant treatment main effect ($F_{treatment} = 4.426$, $p = 0.038$), while no overall effect was observed for either the age main effect or the treatment × age interaction ($F_{age} = 0.474$, $p = 0.624$; $F_{treatment \times age} = 1.633$, $p = 0.271$). Compared with their respective control groups, the AOD of the myelin in the 3WT+ETOH group and the 4WT+ETOH group after alcohol exposure showed no significant differences (Fig. 4A, B, $t = 0.345$, $p > 0.05$; $t = 1.220$, $p > 0.05$). However, the AOD of the myelin decreased by 5.0 % in the 5WT+ETOH group compared to the 5WT+SAL group (Fig. 4A, B, $t = 2.569$, $p < 0.05$).

Second, we compared the number of $CNPase^+$ cells in the mPFC of each group. A multivariate ANOVA suggested a significant treatment main effect, age main effect, and treatment × age interaction ($F_{treatment} = 8.513$, $p = 0.004$; $F_{age} = 18.360$, $p = 0.000$; $F_{treatment \times age} = 7.239$, $p = 0.001$). In 3-week-old WT mice, there was no salient distinction in the count of $CNPase^+$ cells in the mPFC between the 3WT+ETOH group and the 3WT+SAL group after alcohol exposure ($t = -1.458$, $p > 0.05$). However, compared with the respective control groups, the count of $CNPase^+$ cells in the mPFC of the 4WT+ETOH group and the 5WT+ETOH group was significantly reduced by 11.5 % and 23.0 %, respectively (Fig. 4C, D, $t = 3.136$, $p < 0.01$; $t = 2.906$, $p < 0.01$).

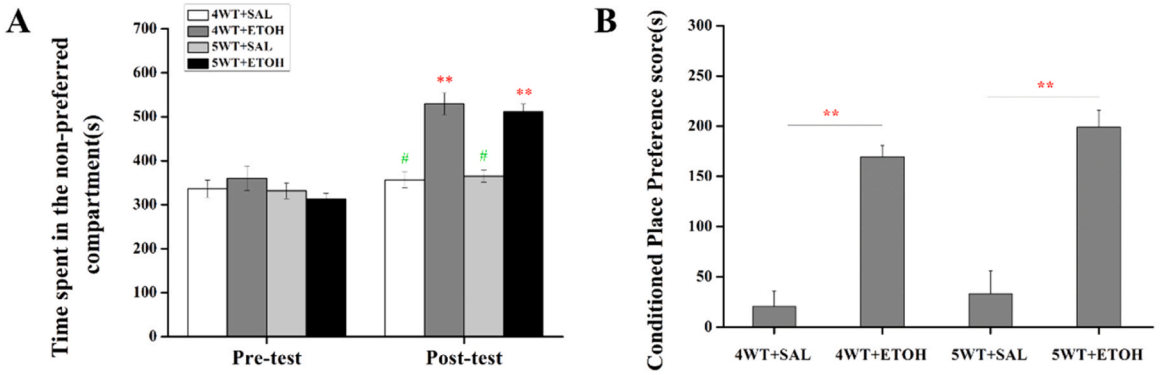

Fig. 2. The effects of alcohol on conditioned place preference in WT mice of various ages. A) Time spent in the non-preferred compartment before and after the conditioning phase. # indicates $p > 0.05$ and ** $p < 0.01$, compared with pre-test. B) CPP scores. Data are presented as mean ± SEM. ** indicates $p < 0.01$, when the CPP acquisition of the 4WT+ETOH group compared with the 4WT+SAL group, and the 5WT+ETOH group compared with the 5WT+SAL group. (n=6 per group).

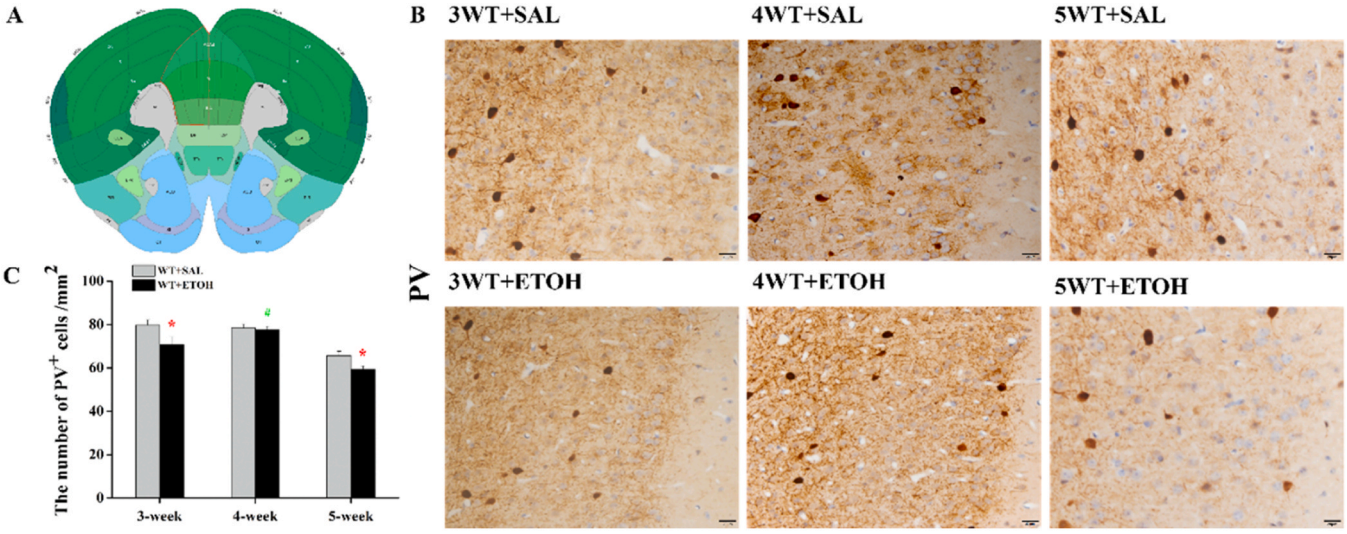

Fig. 3. Alcohol-induced alterations in the number of parvalbumin-positive interneurons in the mPFC of WT mice at different ages. A) Schematic representation of the model of the mPFC brain region. B) The immunohistochemical staining of PV^+ cells in mPFC among the 3WT+SAL group, the 3WT+ETOH group, the 4WT+SAL group, the 4WT+ETOH group, the 5WT+SAL group, and the 5WT+ETOH group. Bar = 20 μm. C) Statistics for PV^+ cell numbers in the mPFC of six groups of mice. Data are presented as mean ± SEM. * indicates $p < 0.05$, and # indicates $p > 0.05$. (n = 18 images, from 6 mice per group).

3.4. Effects of alcohol exposure on OPC numbers in mPFC of WT mice at different ages

OPCs, also known as NG2 glia or polydendrocytes, widely exist in the central nervous system, accounting for about 2 %-9 % of the total cells (Dawson et al., 2003; Nishiyama et al., 2016). The differentiation of OPCs into mature oligodendrocytes capable of myelination is crucial for the normal function of the central nervous system. Expression of neuron-glial antigen 2 (NG2) and platelet-derived growth factor receptor alpha (PDGFRα) as markers of OPCs serves as an indicator of changes in OPC populations (Nishiyama et al., 2021; Zhang et al., 2022). We employed immunohistochemistry and immunofluorescence double-labeling staining to identify alterations in the number of $PDGFR\alpha^+$ cells and $NG2^+/PDGFR\alpha^+$ cells in the mPFC of 3-, 4-, and 5-week-old WT mice after alcohol exposure, respectively.

First, the number of $PDGFR\alpha^+$ cells in mPFC was compared among mice groups. The multivariate ANOVA showed that the main effect of treatment was not significant ($F_{treatment} = 2.669$, $p = 0.105$). However, the main effect of age or the treatment× age interaction was more significant ($F_{age} = 12.075$, $p = 0.000$; $F_{treatment \times age} = 5.883$, $p = 0.004$). Meanwhile, as shown in Fig. 5, the count of $PDGFR\alpha^+$ cells in the mPFC of the 3WT+ETOH group after alcohol exposure was reduced by 6.9 % compared with the 3WT+SAL group ($t = 2.463$, $p < 0.05$). However, there was no significant difference in the count of $PDGFR\alpha^+$ cells between the 4WT+ETOH group and the 4WT+SAL group ($t = -1.640$, $p > 0.05$). In 5-week-old WT mice, the count of $PDGFR\alpha^+$ cells in the mPFC of the 5WT+ETOH group was reduced by 10.5 % compared with the 5WT+SAL group ($t = 2.492$, $p < 0.05$).

Second, we compared the number of $NG2^+/PDGFR\alpha^+$ cells in the mPFC of each group. A multivariate ANOVA suggested a significant treatment main effect ($F_{treatment} = 4.706$, $p = 0.034$), while no overall effect was observed for either the age main effect or the treatment × age interaction ($F_{age} = 1.952$, $p = 0.150$; $F_{treatment \times age} = 0.436$, $p = 0.649$). Meanwhile, as shown in Fig. 6, compared with their respective control groups, the count of $NG2^+/PDGFR\alpha^+$ cells in the mPFC of the 3WT+ETOH group and the 4WT+ETOH group after alcohol exposure showed no significant differences ($t = 0.466$, $p > 0.05$; $t = 1.362$, $p > 0.05$). However, the count of $NG2^+/PDGFR\alpha^+$ cells in the mPFC decreased by 11.4 % in the 5WT+ETOH group compared to the 5WT+SAL group ($t = 2.509$, $p < 0.05$).

In light of a comprehensive comparison of changes in CPP acquisition, myelin, mature oligodendrocytes, and OPC numbers in 3-, 4-, and 5-week-old WT mice following alcohol exposure, subsequent experiments will solely concentrate on 5-week-old WT mice.

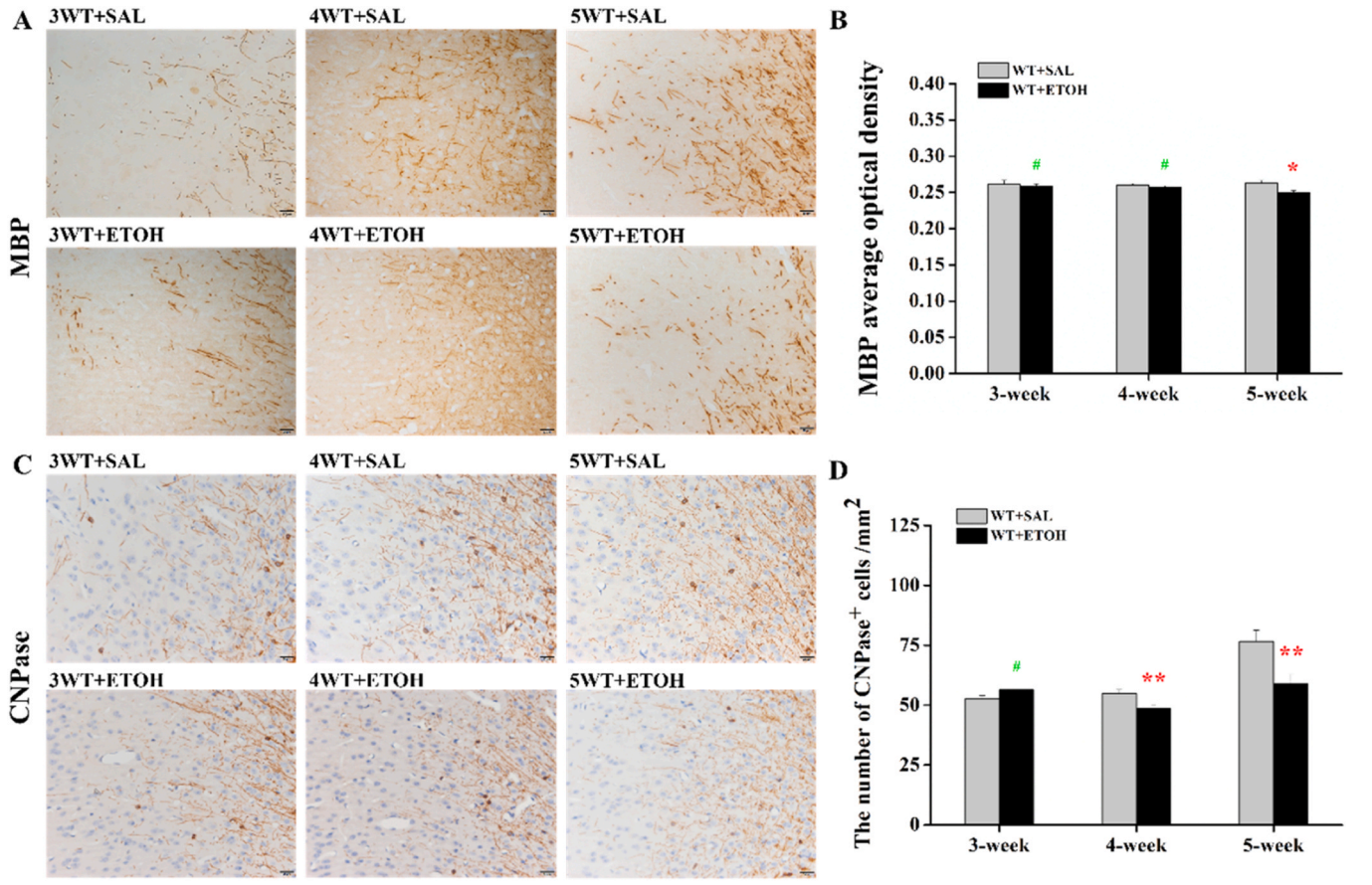

Fig. 4. Alcohol-induced alterations in mature oligodendrocyte numbers and myelin in the mPFC of WT mice at different ages. A) The immunohistochemical staining of MBP in mPFC among the 3WT+SAL group, the 3WT+ETOH group, the 4WT+SAL group, the 4WT+ETOH group, the 5WT+SAL group, and the 5WT+ETOH group. Bar = 20 μm. B) Statistics for the AOD value of MBP in the mPFC of six groups of mice. C) The immunohistochemical staining of CNPase$^+$ cells in mPFC among the 3WT+SAL group, the 3WT+ETOH group, the 4WT+SAL group, the 4WT+ETOH group, the 5WT+SAL group, and the 5WT+ETOH group. Bar = 20 μm. D) Statistics for CNPase$^+$ cell numbers in the mPFC of six groups of mice. Data are presented as mean ± SEM. * indicates $p < 0.05$, ** indicates $p < 0.01$, and # indicates $p > 0.05$. (n = 18 images, from 6 mice per group).

3.5. Effect of alcohol exposure on parvalbumin-positive interneuron numbers in various subregions of mPFC in 5-week-old WT mice

It is widely acknowledged that mPFC is anatomically segregated into ACC, PL, and IL. Each subregion within the mPFC exhibits distinct functional characteristics: the ACC is associated with cognitive activity precision, the PL predominantly engages in emotion-related cognitive processes, and the IL is crucial for impulse control. Moreover, the variability in response patterns of mPFC subregions to external stimuli such as pain, cognitive control, and negative emotion is notable.

Based on the above experiments, we further explored the differences in the count of PV$^+$ interneurons in various subregions of mPFC in 5-week-old WT mice after alcohol exposure. The immunohistochemical staining results are shown in Fig. 7. The multivariate ANOVA showed that the main effect of treatment was significant ($F_{treatment} = 12.382$, $p = 0.001$). However, the main effect of age or the treatment × subregion interaction was more significant ($F_{subregion} = 0.441$, $p = 0.664$; $F_{treatment \times subregion} = 0.362$, $p = 0.697$). Compared with the 5WT+SAL group, the count of PV$^+$ interneurons in the ACC subregion of mPFC in the 5WT+ETOH group after alcohol exposure tended to decrease slightly, but there was no statistical significance ($t = 1.282$, $p > 0.05$). In the PL subregion, the count of PV$^+$ interneurons in the 5WT+ETOH group was reduced by 17.6 % compared with the 5WT+SAL group ($t = 3.042$, $p < 0.01$). In the IL subregion, the count of PV$^+$ interneurons in the 5WT+ETOH group was reduced by 17.2 % compared with the 5WT+SAL group ($t = 2.064$, $p < 0.05$).

3.6. Effect of alcohol exposure on mature oligodendrocyte numbers and myelin in various subregions of mPFC in 5-week-old WT mice

To further explore the differences in the count of mature oligodendrocytes (CNPase$^+$ cells) and the AOD of myelin in the ACC, PL, and IL subregions of mPFC after alcohol exposure in the basis of 5-week-old WT mice. A multivariate ANOVA suggested a significant treatment main effect ($F_{treatment} = 6.664$, $p = 0.011$), while neither the subregion main effect nor the treatment × age interaction was significant ($F_{subregion} = 1.868$, $p = 0.160$; $F_{treatment \times subregion} = 1.441$, $p = 0.241$). In the ACC and PL subregions of mPFC, there was no salient distinction in the AOD of myelin in the 5WT+ETOH group after alcohol exposure compared with the 5WT+SAL group (Fig. 8A, B, $t = 0.101$, $p > 0.05$; $t = 2.106$, $p > 0.05$). In the IL subregion of mPFC, the AOD of myelin in the 5WT+ETOH group decreased by 3.7 % compared with the 5WT+SAL group (Fig. 8 A, B, $t = 2.709$, $p < 0.05$).

The multivariate ANOVA revealed that the treatment main effect was significant ($F_{treatment} = 25.503$, $p = 0.000$), but the subregion main effect and treatment × subregion interaction were not significant ($F_{subregion} = 1.740$, $p = 0.181$; $F_{treatment \times subregion} = 2.084$, $p = 0.130$). In the ACC and IL subregions of the mPFC, CNPase$^+$ cell counts decreased by 29.0 % and 29.0 % in the 5WT+ETOH group compared with the 5WT+SAL group, respectively, following alcohol exposure (Fig. 8C, D, $t = 2.860$, $p < 0.01$; $t = 4.101$, $p < 0.01$). In the PL subregion of mPFC, there was no salient distinction in the number of CNPase$^+$ cells between the 5WT+EtOH group and the 5WT+SAL group after alcohol exposure (Fig. 8C, D, t =

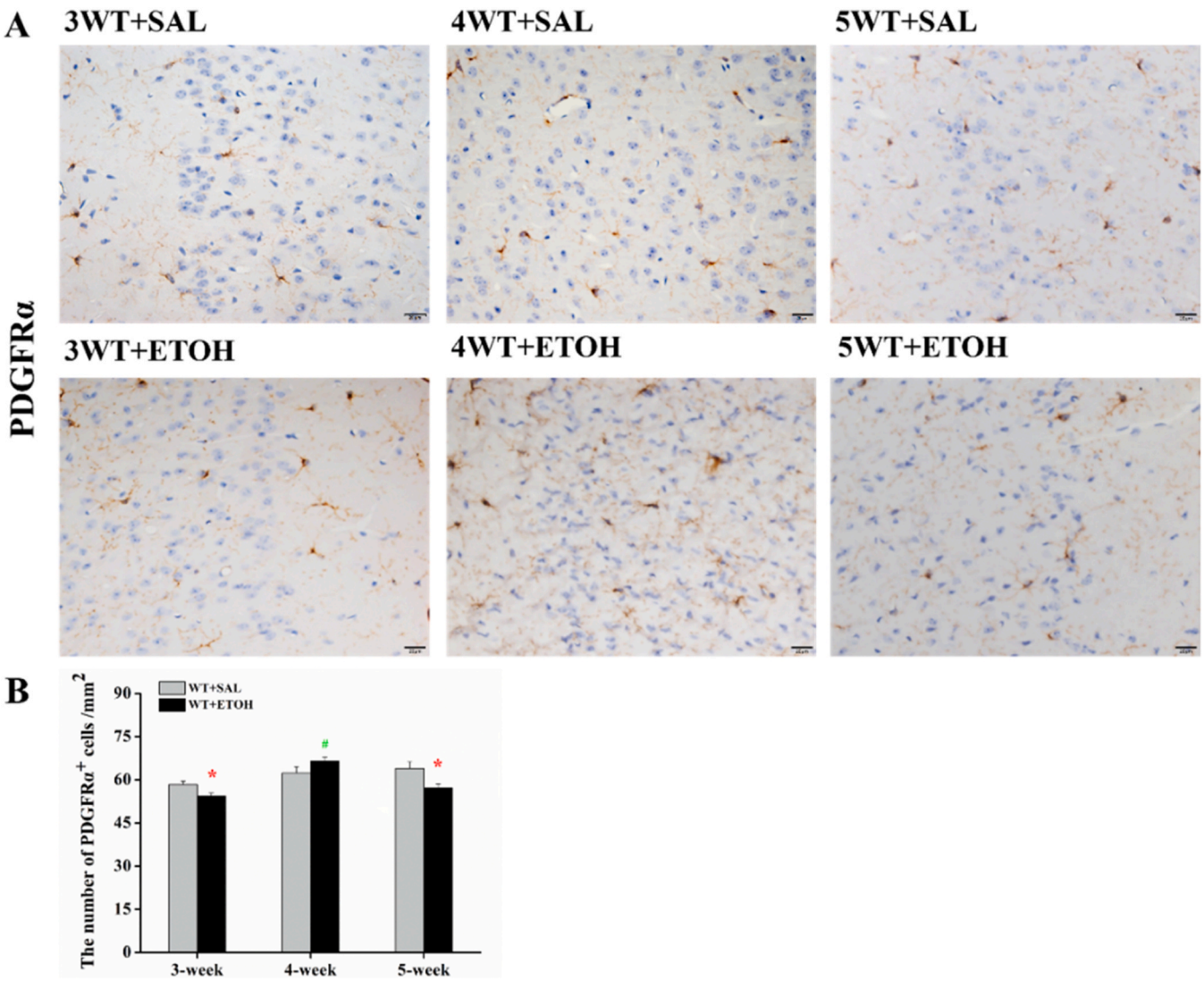

Fig. 5. Alcohol-induced alterations in the number of PDGFRα$^+$ cells in the mPFC of WT mice at different ages. A) The immunohistochemical staining of PDGFRα$^+$ cells in mPFC among the 3WT+SAL group, the 3WT+ETOH group, the 4WT+SAL group, the 4WT+ETOH group, the 5WT+SAL group, and the 5WT+ETOH group. Bar = 20 μm. B) Statistics for PDGFRα$^+$ cell numbers in the mPFC of six groups of mice. Data are presented as mean ± SEM. * indicates $p < 0.05$, and # indicates $p > 0.05$. (n = 18 images, from 6 mice per group).

1.706, $p > 0.05$).

3.7. Effects of alcohol exposure on OPC numbers in various subregions of mPFC in 5-week-old WT mice

The effect of alcohol exposure on the count of PDGFRα$^+$ cells and NG2$^+$/PDGFRα$^+$ cells in ACC, PL, and IL subregions of mPFC in 5-week-old WT mice was further investigated.

First, the number of PDGFRα$^+$ cells in mPFC was compared among mice groups. The multivariate ANOVA revealed that neither the treatment main effect nor the treatment × subregion interaction was significant ($F_{treatment} = 2.102$, $p = 0.150$; $F_{treatment \times subregion} = 3.043$, $p = 0.052$). However, the subregion main effect was more pronounced ($F_{subregion} = 3.619$, $p = 0.030$). Moreover, immunohistochemical results showed that there was no salient distinction in the number of PDGFRα$^+$ cells between the 5WT+ETOH group and the 5WT+SAL group in the ACC and PL subregions of mPFC (Fig. 9, t = 0.621, $p > 0.05$; t = 2.407, $p > 0.05$). In the IL subregion of mPFC, the count of PDGFRα$^+$ cells in the 5WT+ETOH group after alcohol exposure was decreased by 15.3 % compared with the 5WT+SAL group (Fig. 9, t = 3.055, $p < 0.05$).

Second, we compared the number of NG2$^+$/PDGFRα$^+$ cells in the mPFC of each group. A multivariate ANOVA suggested a significant treatment main effect ($F_{treatment} = 10.886$, $p = 0.002$), while no overall effect was observed for either the subregion main effect or the treatment × subregion interaction ($F_{subregion} = 0.864$, $p = 0.426$; $F_{treatment \times subregion} = 0.176$, $p = 0.839$). Moreover, immunofluorescence results showed that there was no salient distinction in the number of NG2$^+$/PDGFRα$^+$ cells between the 5WT+ETOH group and the 5WT+SAL group in the ACC subregion of mPFC (Fig. 10, t = 1.343, $p > 0.05$). In the PL and IL subregions of mPFC, the count of NG2$^+$/PDGFRα$^+$ cells in the 5WT+ETOH group after alcohol exposure was decreased by 17.0 % and 15.5 %, respectively, compared with the 5WT+SAL group (Fig. 10, t = 2.175, $p < 0.05$; t = 2.262, $p < 0.05$).

4. Discussion

Overall, our study compared the acquisition of CPP following alcohol exposure in 3-, 4-, and 5-week-old mice with changes in the number of PV$^+$ interneurons, mature oligodendrocytes, OPCs, and myelin expression in the mPFC. In terms of CPP acquisition, CPP scores increased by 47.0 % and 63.6 % following alcohol exposure in 4- and 5-week-old mice, respectively, with a higher percentage increase in CPP scores in

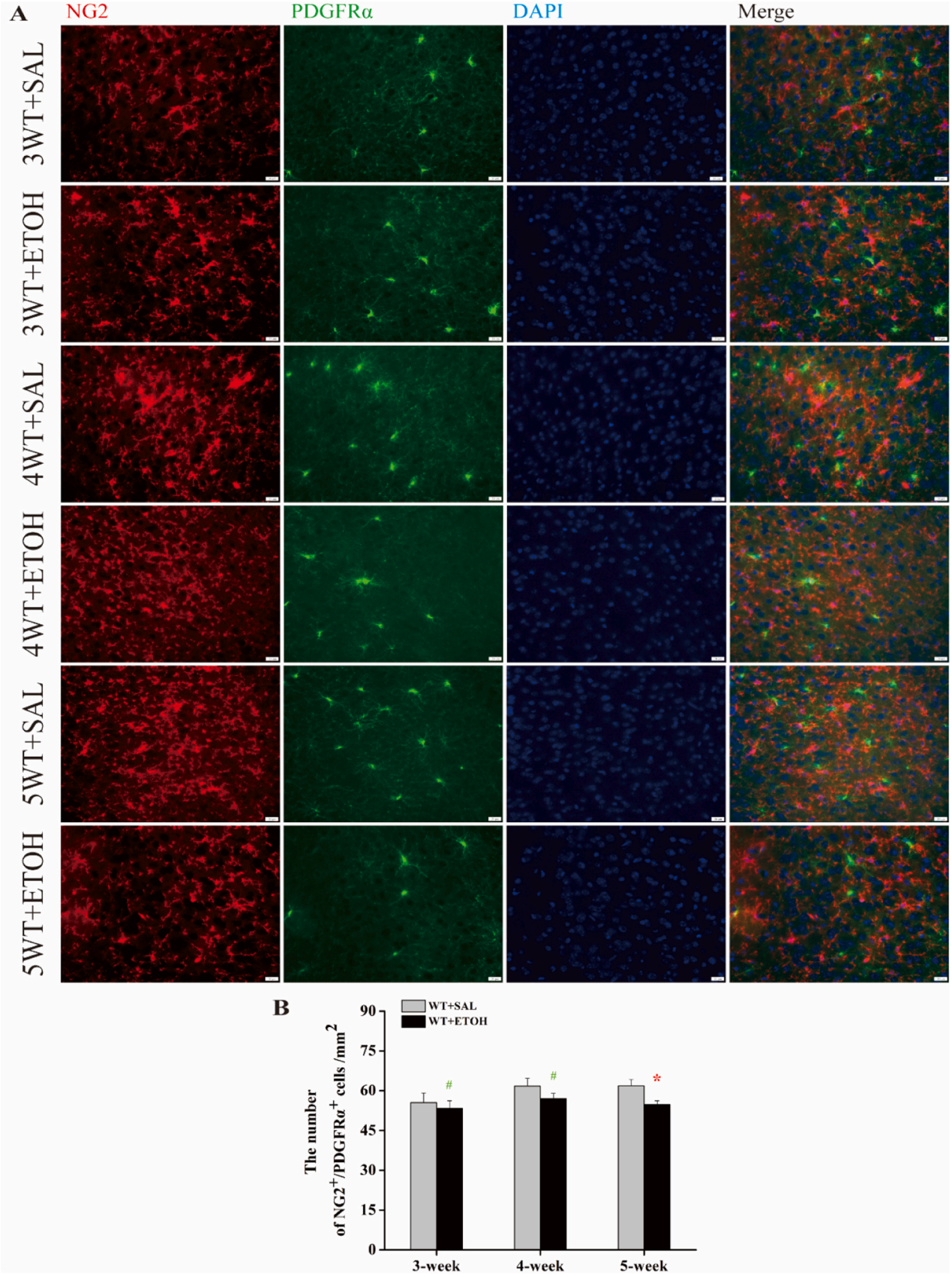

Fig. 6. Alcohol-induced alterations in the number of $NG2^+$/$PDGFR\alpha^+$ cells in the mPFC of WT mice at different ages. A) The immunofluorescence staining of $NG2^+$/$PDGFR\alpha^+$ cells in mPFC among the 3WT+SAL group, the 3WT+ETOH group, the 4WT+SAL group, the 4WT+ETOH group, the 5WT+SAL group, and the 5WT+ETOH group. Bar = 20 μm. B) Statistics for $NG2^+$/$PDGFR\alpha^+$ cell numbers in the mPFC of six groups of mice. Data are presented as mean ± SEM. * indicates $p < 0.05$, and # indicates $p > 0.05$. (n = 12 images, from 6 mice per group).

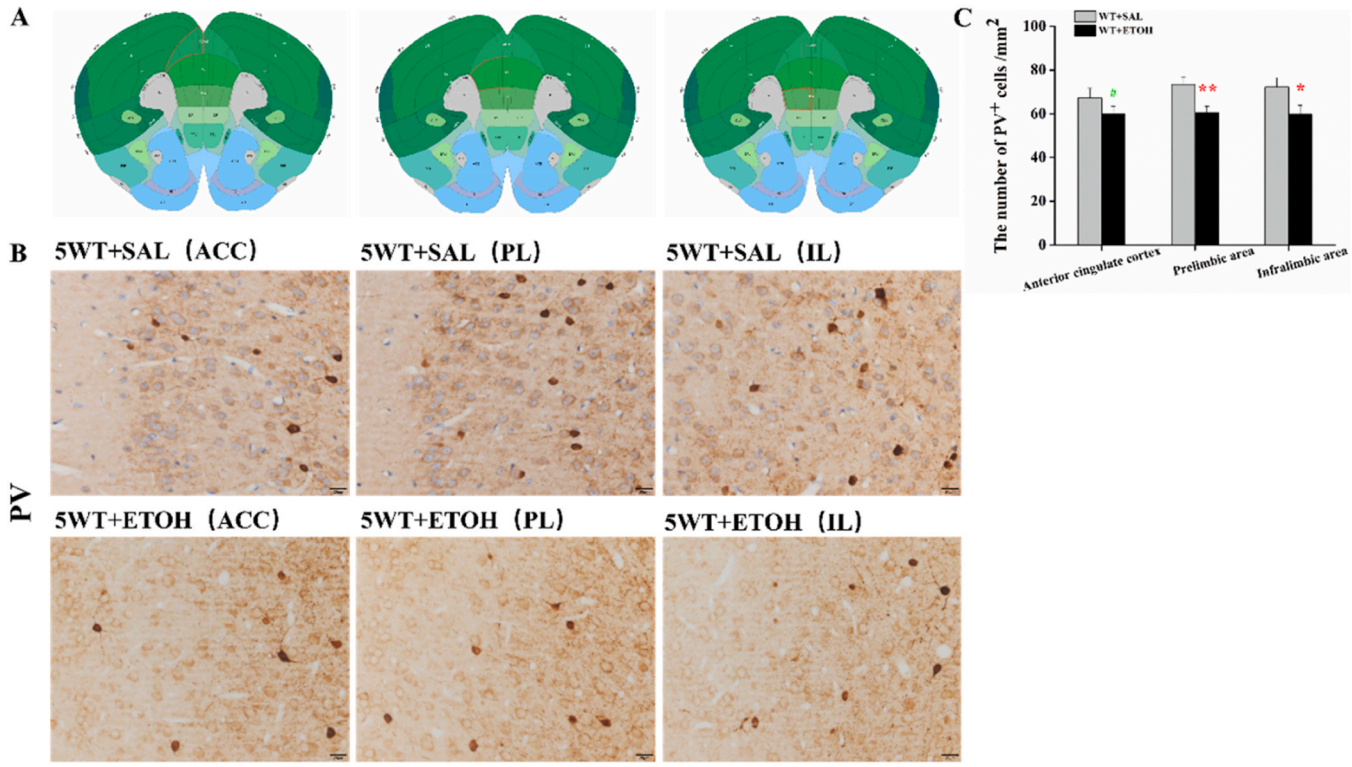

Fig. 7. Alcohol-induced alterations in the number of parvalbumin-positive interneurons in various mPFC subregions in 5-week-old WT mice. A) Schematic representation of the model for the three subregions in the mPFC. B) The immunohistochemical staining of PV^+ cells in the ACC, PL, and IL subregions of mPFC in the 5WT+SAL and 5WT+ETOH groups. Bar = 20 μm. C) Statistics for PV^+ cell numbers in the ACC, PL, and IL subregions of mPFC in the 5WT+SAL and 5WT+ETOH groups. Data are presented as mean ± SEM. * indicates $p < 0.05$, ** indicates $p < 0.01$, and # indicates $p > 0.05$. (n = 18 images, from 6 mice per group).

5-week-old mice. Similarly, alcohol exposure reduced the number of mature oligodendrocytes in mPFC by 11.5 % and 23.0 % in 4- and 5-week-old mice, respectively, with a higher reduction in mature oligodendrocyte numbers in 5-week-old mice. Only 5-week-old mice showed myelin disruption in the mPFC following alcohol exposure. The number of PV^+ interneurons and OPCs in the mPFC decreased by 10.0 % and 10.5 %, respectively, following alcohol exposure in 5-week-old mice, whereas the difference was not significant in 4-week-old mice. As shown, 5-week-old mice were more significantly affected by alcohol exposure than 3- and 4-week-old mice. The mPFC subregion was further explored by targeting the mPFC subregion in alcohol-exposed 5-week-old mice. In terms of myelin expression and changes in the number of OPCs, only the IL subregion showed a significant decrease of 3.7 % and 15.3 %, respectively. The number of mature oligodendrocytes in the ACC and IL subregions was significantly reduced. Similarly, the number of PV^+ interneurons in the PL and IL subregions decreased significantly. Alcohol exposure appears to have the greatest effect on the IL subregion of the mPFC in 5-week-old mice.

The CPP model, an internationally recognized and well-validated animal model, is extensively employed to evaluate the psychiatric dependence properties of alcohol. Our findings indicate a significant acquisition of CPP in 4- and 5-week-old WT mice post alcohol exposure, aligning with previous research that confirmed the potent acquisition of CPP in adolescent mice due to alcohol exposure (Carrara-Nascimento et al., 2014). Furthermore, Romero-Torres et al. (Romero-Torres et al., 2023) suggested that adolescent rats, post alcohol exposure, exhibit heightened compulsive and voluntary drinking behavior alongside a pronounced alcohol-CPP. In the current study, 3-week-old WT mice, post alcohol or saline treatment, lingered excessively in one compartment of the CPP box, disqualifying them from the CPP experiment. This phenomenon might be attributed to several factors: firstly, the neurobiological mechanisms tied to environmental or drug-triggered sensitization responses are typically not fully developed until 3–4 weeks after birth (Scalzo & Holson, 1992; Tsuchida et al., 1994; Ujike et al., 1995); secondly, the mice's underdeveloped locomotor abilities due to their young age. Studies (Tirelli, 2001a; b; Tirelli & Ferrara, 1997) highlight that various indicators, besides locomotion, such as uncoordinated movements in 1-week-old pups, persisted wall-climbing in 8–12 day-old pups, and adult-like head-scanning movements in 3-week-old pups, signify sensitization; thirdly, visible human interventions (e.g., intraperitoneal injection) during CPP procedures may inadvertently influence the mice's behavioural performance.

Research has indicated a robust association between alcohol dependence and brain impairments, especially impacting executive and cognitive functions in the mPFC (Koob, 2013). The PV^+ interneurons within the mPFC have a critical role in maintaining the excitatory/inhibitory balance, which is essential for the functional rhythms and activity of brain networks (Cho et al., 2015). Notably, alterations in cognitive function have been closely linked to impaired PV^+ interneurons (Ruden et al., 2021). Our findings show that after alcohol exposure, the number of PV^+ interneurons in the mPFC of both 3- and 5-week-old WT mice was lower than in their control groups, with a minor trend toward a decline in the count of PV^+ interneurons in the mPFC of 4-week-old WT mice. Consistent with a previous study, developmental alcohol exposure results in a decrease in the count of PV^+ interneurons in the mPFC, thereby causing impaired executive function in the mPFC (Hamilton et al., 2017). Liu and colleagues (Liu & Crews, 2017) demonstrated that the number of PV^+ interneurons in the hippocampal dentate gyrus and subventricular zone of the forebrain was reduced in adolescent Wistar rats after chronic intermittent alcohol vapor exposure and persisted into adulthood. It has also been reported that adolescent rodents receiving intermittent alcohol exposure show no changes in PV^+ interneurons in the prefrontal cortex after adulthood but an increase in their surrounding extracellular perineuronal nets. An increase in perineuronal nets restricts the plasticity of the PV^+ interneurons, which leads to associated behavioral deficits (Dannenhoffer

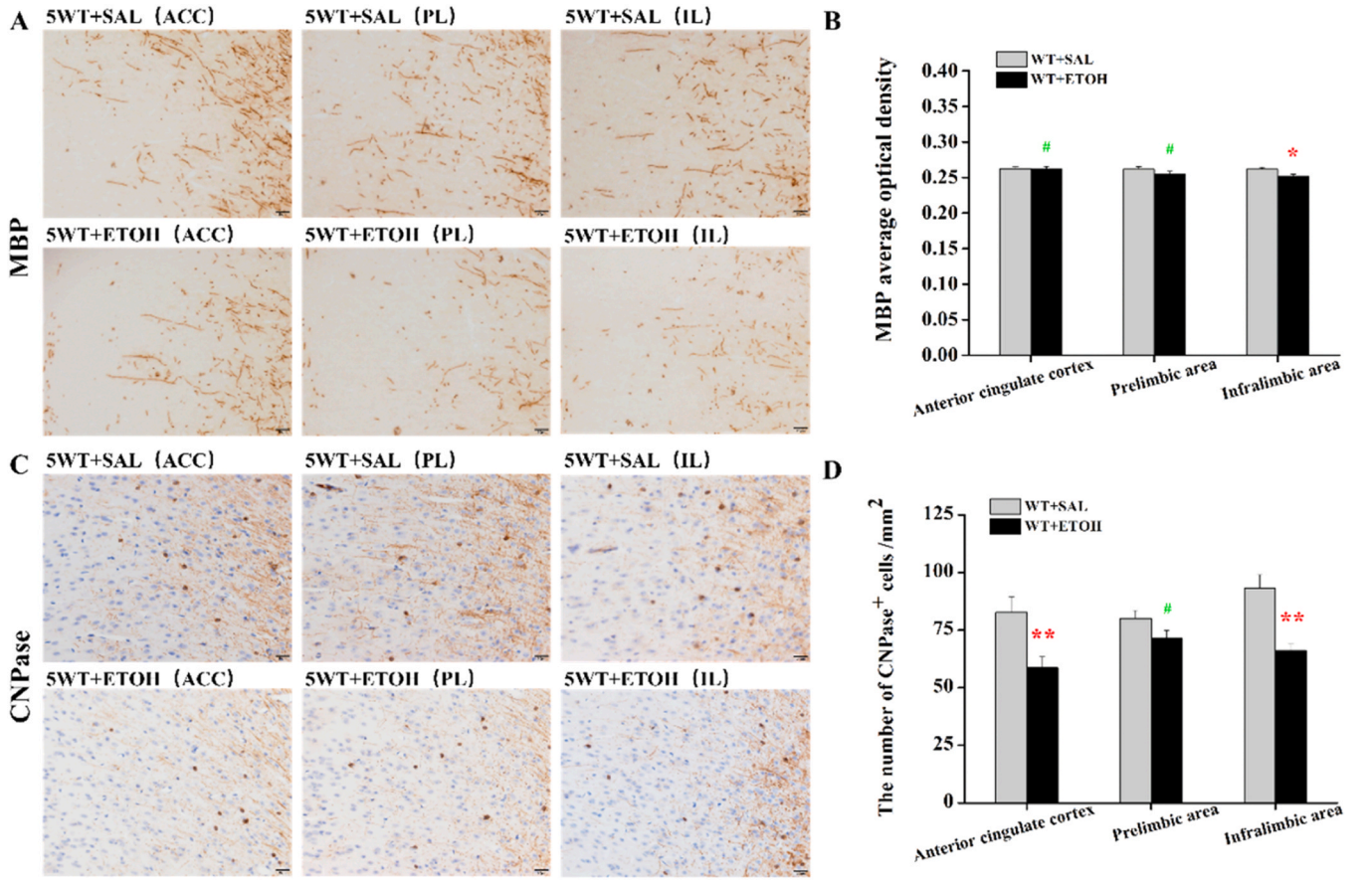

Fig. 8. Alcohol-induced alterations in mature oligodendrocyte numbers and myelin in various mPFC subregions in 5-week-old WT mice. A) The immunohistochemical staining of MBP in the ACC, PL, and IL subregions of mPFC in the 5WT+SAL and 5WT+ETOH groups. Bar = 20 μm. B) Statistics for the AOD value of MBP in the ACC, PL, and IL subregions of mPFC in the 5WT+SAL and 5WT+ETOH groups. C) The immunohistochemical staining of CNPase$^+$ cells in the ACC, PL, and IL subregions of mPFC in the 5WT+SAL and 5WT+ETOH groups. Bar = 20 μm. D) Statistics for CNPase$^+$ cell numbers in the ACC, PL, and IL subregions of mPFC in the 5WT+SAL and 5WT+ETOH groups. Data are presented as mean ± SEM. * indicates $p < 0.05$, ** indicates $p < 0.01$, and # indicates $p > 0.05$. (n = 18 images, from 6 mice per group).

et al., 2022).

Myelin, a multilayered membrane structure, is formed by the helical wrapping and compaction of the oligodendroglial plasma membrane surrounding axons in the central nervous system. This configuration enables myelinated nerve fibers to transmit electrical signals with both speed and efficiency (Simons & Nave, 2015). Myelin remodeling persists in the central nervous system of adult mice. This process mainly occurs due to myelin degeneration and an elevation in newly generated oligodendrocytes and myelin (Hughes et al., 2018; Wang et al., 2020a). The destruction of myelin severely affects the structure of the mPFC and its function in fear learning and memory (Navarro, &Mandyam, 2015; Pan et al., 2020). Our study revealed alcohol exposure-induced variations in the AOD values of myelin in the mPFC in WT mice of different ages (P21, P28, and P35). The 5-week-old WT mice were particularly impacted by alcohol exposure, evidencing significant changes. Concurrently, alcohol exposure notably reduced the count of oligodendrocytes in the mPFC of 4- and 5-week-old WT mice, whereas no significant abnormality was observed in mPFC oligodendrocyte counts in 3-week-old WT mice. Corroborating Newville et al. (Newville et al., 2017), postnatal day 1 (P1) mice undergoing two weeks of alcohol exposure manifested reduced MBP expression in the corpus callosum, a 58 % reduction in mature oligodendrocyte numbers, with MBP impairment persisting until P50. Although not similarly reported, McDougall et al. showed that between pre-adolescence and mid-adolescence (P15–P43), a large number of axons projecting from the anterior branches of the corpus callosum to neurons in layer V in the ACC subregion of the mPFC are encapsulated by myelin (McDougall et al., 2018). During this period, the number of myelinated axons increased almost 90-fold.

OPCs widely exist throughout the central nervous system and are capable of differentiating into mature oligodendrocytes, which are responsible for myelin generation. The age-dependent decrease in myelin regeneration potential in the central nervous system is closely related to the decline in OPCs differentiation capacity (Ma et al., 2022). The failure of remyelination caused by cell death or lack of differentiation of OPCs can result in severe neurological impairments and motor dysfunction (Shen et al., 2022). Our data indicated that alcohol significantly decreased the number of OPCs in the mPFC in 3- and 5-week-old WT mice, whereas a small, but not significant, evaluation in the count of OPCs in the mPFC was detected in 4-week-old WT mice after alcohol exposure. Consistent with our report, 4-week-old mice had a salient reduction in OPCs in the mPFC after 10 days of social failure (Chen et al., 2022). Newville and colleagues also suggested that the count of OPCs in the corpus callosum of P16 mice was reduced by 75 % when mice pups were exposed to alcohol for 2 weeks (Newville et al., 2017). In rodents, chronic intermittent alcohol vapor exposure not only suppresses the length of the S-phase of the glial progenitor cell cycle but also decreases the proliferation and differentiation of glial progenitor cells into OPCs (Kim et al., 2015). In addition, some reports suggested a large increase in OPCs in the corpus callosum forceps after intermittent alcohol exposure (P25-P54) in adolescent Wistar rats and persisted until P95 (Liu et al., 2021). In response to stimulation by various cytokines, growth factors, or neuronal activation, OPCs proliferation increases (Moyon et al.,

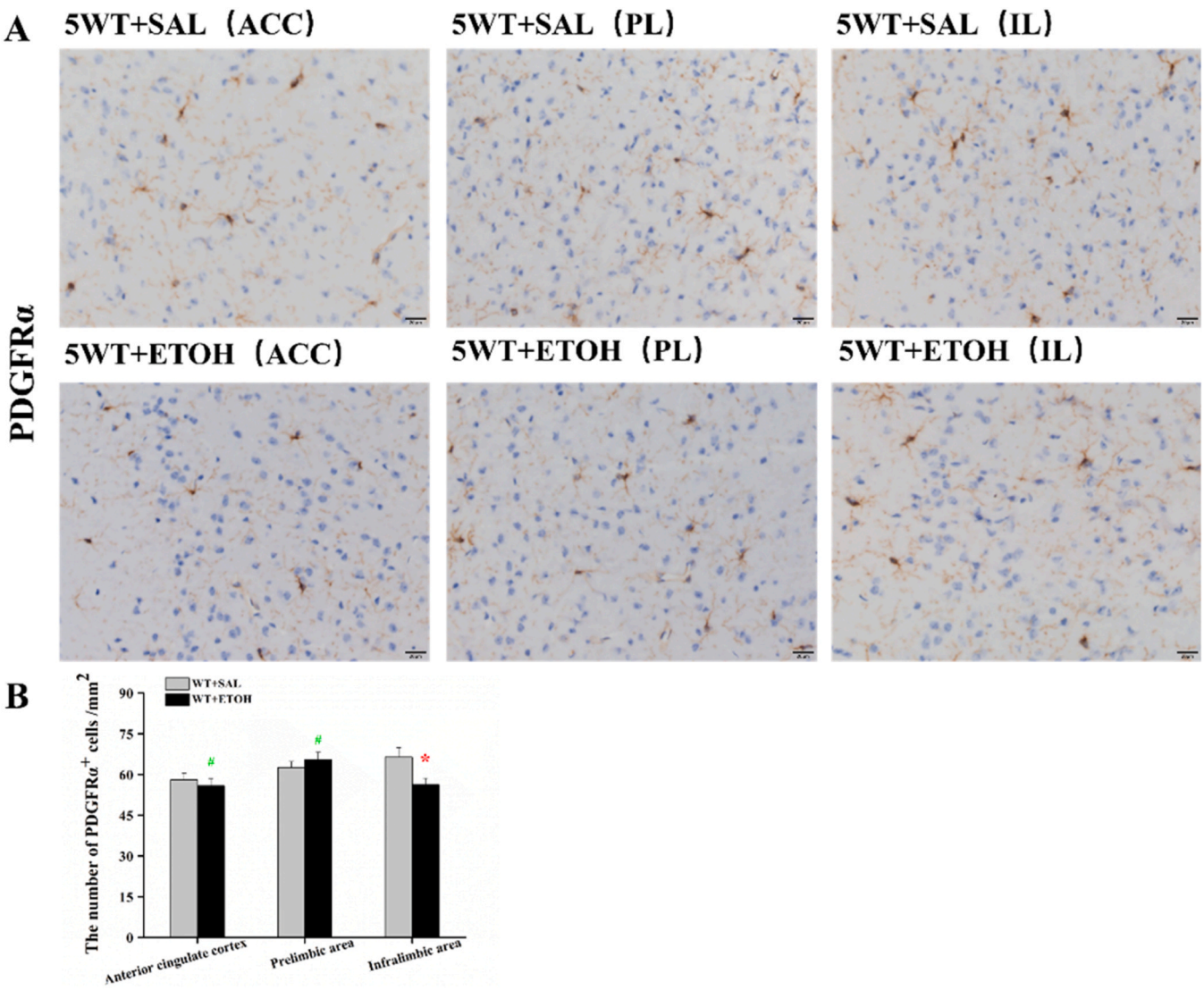

Fig. 9. Alcohol-induced alterations in the number of PDGFRα$^+$ cells in various mPFC subregions in 5-week-old WT mice. A) The immunohistochemical staining of PDGFRα$^+$ cells in the ACC, PL, and IL subregions of mPFC in the 5WT+SAL and 5WT+ETOH groups. Bar = 20 μm. B) Statistics for PDGFRα$^+$ cell numbers in the ACC, PL, and IL subregions of mPFC in the 5WT+SAL and 5WT+ETOH groups. Data are presented as mean ± SEM. * indicates $p < 0.05$, and # indicates $p > 0.05$. (n = 18 images, from 6 mice per group).

2016). Reactive proliferation also occurs following exposure to alcohol (He et al., 2009).

It is well known that the medial prefrontal cortex in rodents can be subdivided into ACC, PL, and IL. It has been shown that the three different subareas of the mPFC have diverse functions (Capuzzo, &Floresco, 2020; Dalley et al., 2004; Rolls, 2019; Tzschentke, &Schmidt, 1999) and exhibit varying degrees of heterogeneity in response to disturbances from numerous external factors (Tzschentke, &Schmidt, 1999; Yashima et al., 2023). Specifically, our results showed that the PV$^+$ interneuron numbers in the PL and IL subregions all decreased to varying degrees, and the PV$^+$ interneuron numbers in the ACC subregion also exhibited a decreasing trend. These data extend the literature on the negative impacts of alcohol exposure in adolescent mice on the expression of PV$^+$ interneurons and support a subregion-specific effect in the mPFC. For example, Sparta and colleagues suggested that an increase in the activity of PV$^+$ interneurons in the PL subregion inhibits the excitatory mPFC network, thereby promoting the extinction of significant acquired cue reward responses (Sparta et al., 2014). Likewise, following exposure to intoxicating doses (2 g/kg) of alcohol, mice displayed CPP linked to decreased excitability of PV$^+$ interneurons in the PL subregion (Ferranti et al., 2022). Meanwhile, a study indicated different results that developmental alcohol exposure affected the count and volume of PV$^+$ interneurons in the ACC region, but the PL, IL subregions of the mPFC were unaffected (Hamilton et al., 2017). There was a 45 % decrease in PV+ interneurons in the ACC of alcohol-exposed rats antepartum (Moore et al., 1998). In conclusion, the differences in PV$^+$ interneuron expression in different subregions of the mPFC caused by alcohol exposure may be closely related to the animal strain, the duration, frequency, and dosage of alcohol exposure, as well as the unique susceptibility of each subregion to alcohol.

Our results indicated that in 5-week-old WT mice exposed to alcohol, AOD values of myelin in the ACC subregion of the mPFC were diminished compared to controls. In contrast, the AOD values of myelin in the PL and IL subregions were not markedly altered. Meanwhile, the number of oligodendrocytes in the ACC and IL subregions of the alcohol-exposed mice was significantly lower compared to controls, whereas oligodendrocytes in the PL subregion tended to decrease only slightly. As a result, 5-week-old WT mice showed significant heterogeneity in changes in myelin and mature oligodendrocytes in all three subregions of the mPFC after alcohol exposure. A similar report using diffusion tensor imaging

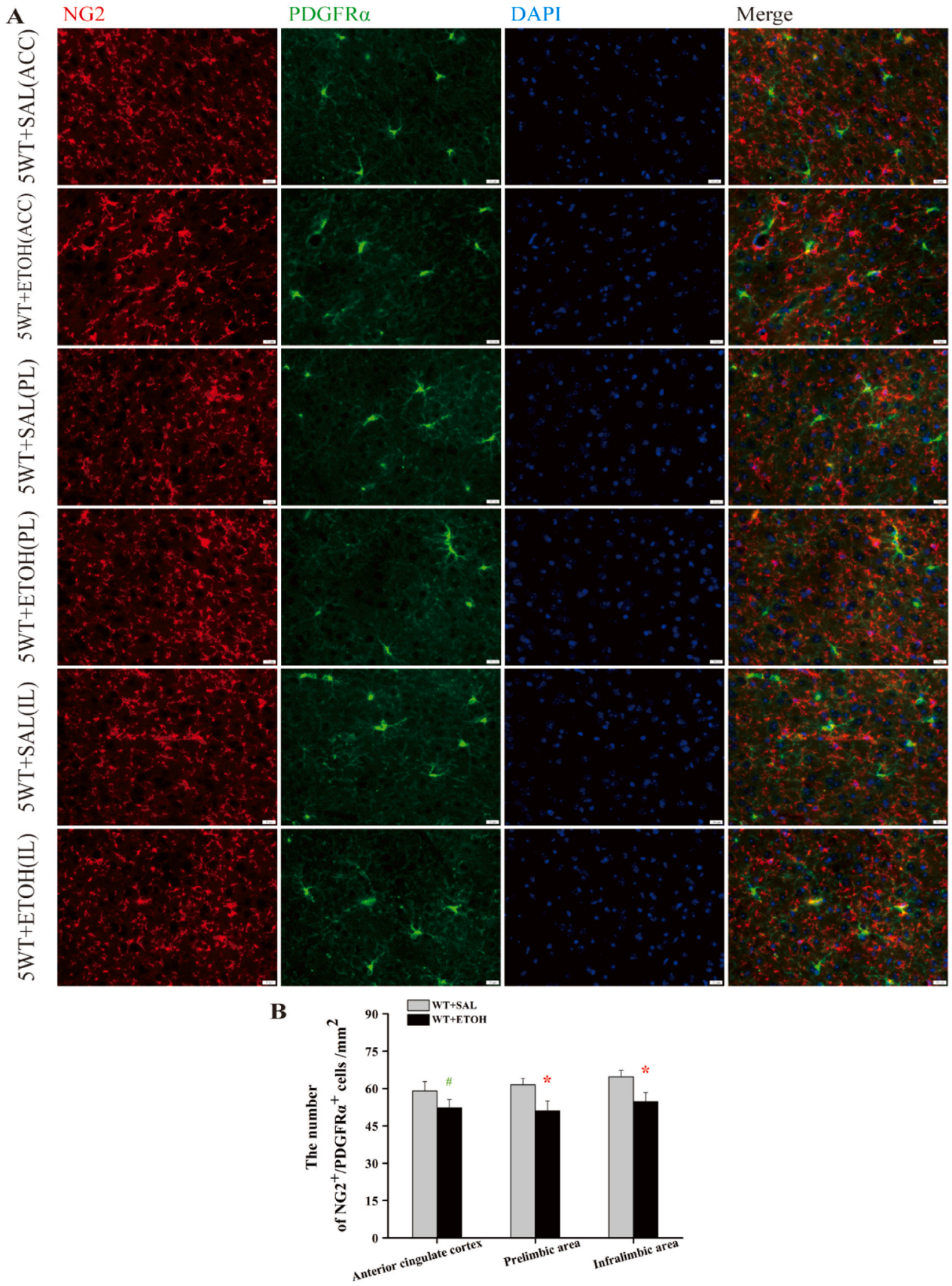

Fig. 10. Alcohol-induced alterations in the number of $NG2^+/PDGFR\alpha^+$ cells in various mPFC subregions in 5-week-old WT mice. A) The immunofluorescence staining of $NG2^+/PDGFR\alpha^+$ cells in the ACC, PL, and IL subregions of mPFC in the 5WT+SAL and 5WT+ETOH groups. Bar = 20 μm. B) Statistics for $NG2^+/PDGFR\alpha^+$ cell numbers in the ACC, PL, and IL subregions of mPFC in the 5WT+SAL and 5WT+ETOH groups. Data are presented as mean ± SEM. * indicates $p < 0.05$, and # indicates $p > 0.05$. (n = 12 images, from 6 mice per group).

identified specific microstructural integrity disruptions in seven subregions of the corpus callosum in alcohol-dependent patients, mainly manifested as axonal and myelin modifications in the isthmus of the corpus callosum (Wang et al., 2019). Moreover, alcohol dependence is linked to pronounced declines in lipid expression, closely associated with myelin, in the frontal white matter, displaying regional disparities (de la Monte et al., 2018). Prenatal alcohol exposure, however, did not impact myelin formation in corticospinal axons across rats of varying ages (Miller, 2017). There were also discernible disparities in alcohol-induced gene expression regulation in different regions between DBA/2 J and C57BL/6 J mice (Kerns et al., 2005). Liu et al. (Liu et al., 2021) found that intermittent alcohol exposure in adolescent Wistar rats influenced oligodendrocyte expression across three prefrontal cortex regions: PLP^+, MBP^+, MAG^+, MOG^+, $CNPase^+$, $Olig1^+$, and $Olig2^+$, yet with a distinct regional and marker specificity. Separately, a non-alcohol-related report found that in three stress-related regions of the brains of adolescent mice exposed to the stress of social failure, there were no significant differences in APC^+ mature oligodendrocytes in all brain areas between stressed mice and controls, except for a reduction in APC^+ mature oligodendrocytes in the PL subregion of the mPFC in stressed mice (Chen et al., 2022). In summary, brain region-specific changes in signaling and neuronal plasticity may be key factors in the development of enduring behavioral phenotypes (e.g., dependence, sensitivity, and craving) (Kerns et al., 2005).

Moreover, post alcohol exposure in 5-week-old WT mice resulted in a notable decrease in the count of OPCs in the IL subregion compared to the control group. Although the number of OPCs in the ACC subregion showed a reduction trend, the distinction was not salient compared to the control group. A slight, non-salient rise was detected in the PL subregion. This data underscores the influence of adolescent alcohol exposure on the subregional specificity of OPCs expression. In synthesizing these observations, we attribute these trends to factors such as the age of the mice, the duration of alcohol exposure, and the reactive proliferation and differentiation of OPCs. A congruent study reported a substantial increase in OPCs in the corpus callosum forceps following intermittent alcohol exposure in adolescent rats, presenting different OPCs trends in the PL and IL regions (Liu et al., 2021). The reactive proliferation of OPCs, stimulated by alcohol, cytokines, or neuronal activation, somewhat counterbalances the harm or interference of various deleterious factors (He et al., 2009; Moyon et al., 2016). During development, OPCs tends to proliferate more expediently in the white matter (e.g., corpus callosum) than in the gray matter (Psachoulia et al., 2009; Young et al., 2013), further highlighting the variability of susceptibility in individual experimental animals.

We must emphasize that the effects of alcohol exposure on CPP acquisition in mice of different ages were pronounced in our study, whereas the changes in the number of PV^+ interneurons, mature oligodendrocytes, and OPCs, especially myelin expression in the mPFC were relatively slight. First, the lack of parallelism between behavioral phenotypic changes and alterations in myelin or oligodendrocytes within the mPFC may stem from differences in time, frequency, and dose of alcohol exposure, with low alcohol concentrations or brief exposures likely to result in only minor myelin changes. Guo et al. exposed adolescent mice to 5 % or 10 % alcohol for 3 consecutive weeks and discovered reductions in oligodendrocytes and myelin in the prefrontal cortex and corpus callosum, but no significant effect on overall MBP expression (Guo et al., 2021). Similarly, our study's CPP behavioral trial lasted 12 days, including 4 alcohol exposures, and the effect on MBP expression was relatively late and mild. Second, adolescent mice's brains are rapidly developing and remodeling, particularly myelination of the cortex and limbic system (Makinodan et al., 2012). Thus, the slight alterations in myelination observed in our studies may stem from the combined effects of myelin formation disturbance and natural development and remodeling. Third, alcohol exposure causing significant CPP acquisition in adolescent mice is a complex process involving the interaction of various molecular and cellular pathways, and slight alterations to myelin may be just one of the explanations (Ben Hamida et al., 2018; Liu et al., 2020). However, this slight difference still warrants further investigation and exploration to fully understand the effects of alcohol exposure on brain development and function. In the motor cortex of adolescent mice exposed to 20 % ethanol for 7 consecutive days, Shi-Yu Peng et al. found only reduced firing frequency in layer 5 vertebral neuronal cells adjacent to the mPFC (Peng et al., 2021). PV^+ interneurons, as the main GABAergic neurons in the mPFC (Perova et al., 2015), may also be present in our study only in terms of altered neuronal firing frequency, while changes in the number of PV^+ interneurons have not yet occurred.

5. Conclusions

In conclusion, our study unprecedentedly unveils that post alcohol exposure to 3-, 4-, and 5-week-old adolescent mice, differences in CPP acquisition, the count of PV^+ interneurons, OPCs, and oligodendrocytes, and the impairment of myelination in the mPFC were more pronounced in 5-week-old adolescent mice. Particularly in 5-week-old adolescent mice, these changes in the IL subregion of the mPFC were most substantially affected by alcohol exposure. Given the crucial role of the IL subregion in active and inhibitory avoidance as well as in the curtailment of reward-seeking behaviors, further exploratory studies regarding the relationship between this brain area and deficits in alcohol-exposed brain function may result in the development of more targeted interventions to mitigate alcohol-induced impairments in the mPFC.

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Ethical statement

The experimental design, experimental process and animal execution method were all in line with the requirements of the Ethical Review Committee for experimental animal welfare of the Army Medical University (AMUWEC202051).

CRediT authorship contribution statement

Xiaolong Li: Methodology, Formal analysis. **Hongli Zhou:** Methodology, Conceptualization. **Yan Cai:** Methodology, Conceptualization. **Zuo Zhang:** Project administration, Methodology, Conceptualization. **Jiyin Zhou:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Maolin Li:** Writing – review & editing, Methodology, Data curation. **Zhifei Qiao:** Investigation, Formal analysis, Data curation. **Dong Huang:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

There is no conflict of interest to declare.

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