EARLY LIFE STRESS-INDUCED EPIGENETIC CHANGES INVOLVED IN MENTAL DISORDERS

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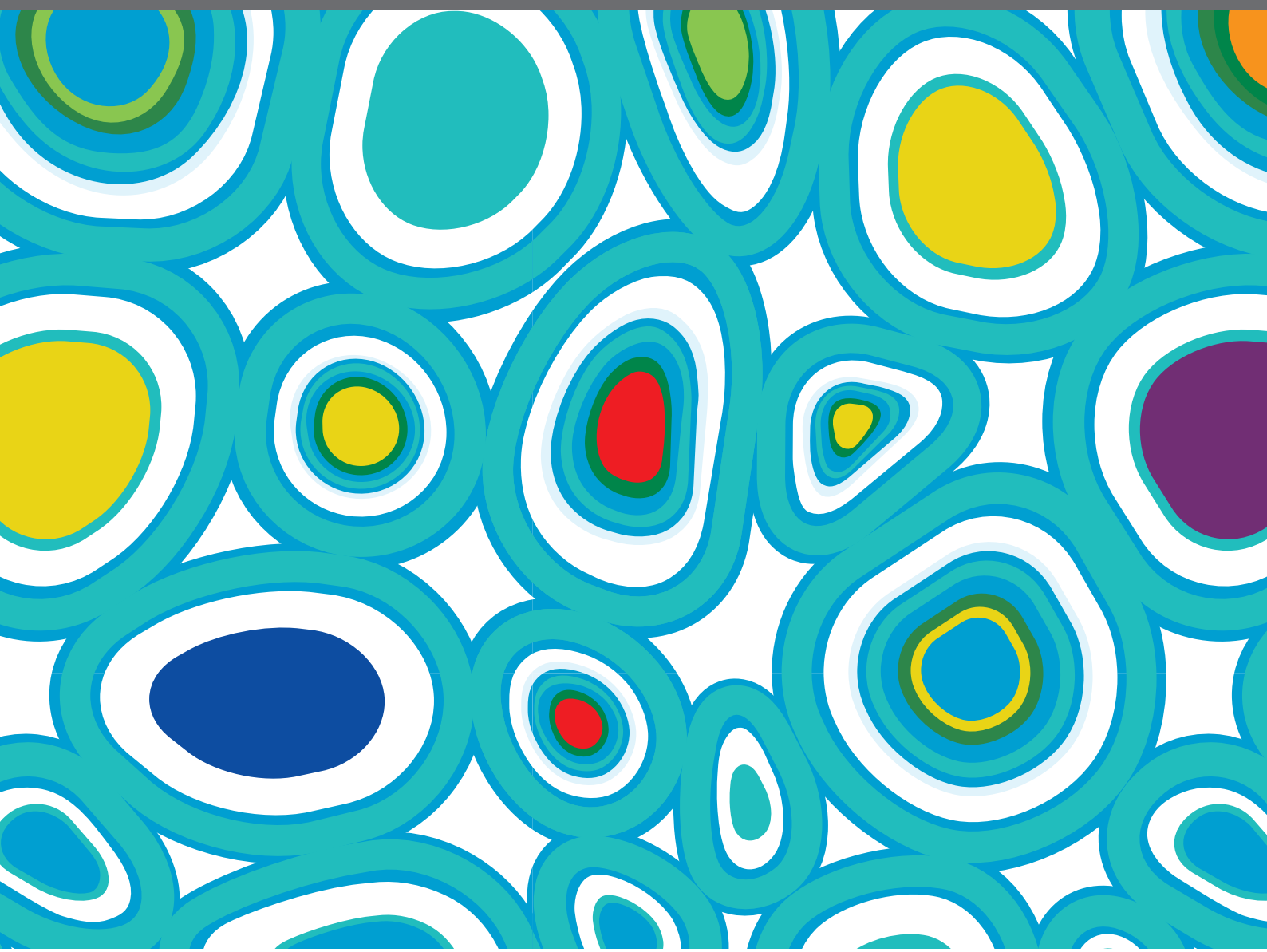

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EARLY LIFE STRESS-INDUCED EPIGENETIC CHANGES INVOLVED IN MENTAL DISORDERS

Topic Editors:
Fushun Wang, Nanjing University of Chinese Medicine, China
Jason H. Huang, Baylor Scott and White Health, United States
Fang Pan, Shandong University, China
Yi-Yuan Tang, Texas Tech University, United States

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Editorial: Early Life Stress-Induced Epigenetic Changes Involved in Mental Disorders

Fushun Wang[1]*, *Fang Pan*[2], *Yiyuan Tang*[3] and *Jason H. Huang*[4,5]

[1] Institute of Brain and Psychological Sciences, Sichuan Normal University, Chengdu, China, [2] Department of Medical Psychology, Shandong University Medical School, Jinan, China, [3] Department of Psychological Sciences, Texas Technological University, Lubbock, TX, United States, [4] Department of Neurosurgery, Baylor Scott & White Health, Temple, TX, United States, [5] Department of Surgery, Texas A&M University College of Medicine, Temple, TX, United States

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Editoial on the Research Topic

Early Life Stress-Induced Epigenetic Changes Involved in Mental Disorders

Early-life stress is known to be a leading cause of mental illness, and it is a form of chronic social stress that has long-lasting consequences on mental health. However, how exposure to stress during a critical period of development may produce severe long-term neurological changes is still not clear. Epigenetic processes may be one way by which early-life stress becomes biologically embedded, altering how children respond physiologically and behaviorally to stress. The application of epigenetic principles to developmental science is an area of research that has received a great deal of attention in recent years. Some of the most important evidence for epigenetic mediators of early life stems from the work of Brown et al. (2008) who found that early life stress facilitates changes in the cellular methylome at the glucocorticoid receptor gene (Nr3c1) and that these methylations differences persist until adulthood (Weaver et al., 2006). Weaver et al. (2006) provided a mechanistic basis for understanding the down-regulation of the corresponding Nr3c1 mRNA and protein and the attenuated response to subsequent stressors (Brown et al., 2008). Moreover, they have subsequently shown that these differences in DNA methylations are associated with an increased risk for psychiatric disorders later in life. Although new, epigenetics promise to advance the field of child development and research on the effects of early-life stress in several important ways, This is a fast-growing field, and more recent studies have found that several epigenetic mechanisms, including DNA methylation, histone modifications, and miRNA, may play a dynamic physiological role in the adult brain (Kumar and Mohapatra, 2021).

The idea that early life stress leads to mental health problems dates back to Freud, who proposed that psychosis occurs due to traumatic events experienced in early life that are remembered in the subconscious mind. Later on, Bowlby found that early life pressure could cause changes in social relationships, which had been involved in many psychological problems. Bowlby called this relationship attachment, which would affect social relationships in adulthood. Attachment can be divided into guaranteed attachments and non-guaranteed attachments, which could lead to tensions that would cause mental disorders such as phobia, anxiety, or depression (Gu et al., 2016). While the negative effects of early-life stress on children's developmental outcomes are well documented, we know little about how these processes unfold and which children are more susceptible to these exposures. More recently, many investigators have attempted to evaluate the long-term neurological or behavioral changes caused by the stress of early life (Craig et al., 2021). Numerous preclinical and clinical studies show a strong correlation between the HPA axis and the monoaminergic system with aberrant mental health (Wang et al., 2020). Early life stress usually

Edited by:
Mojgan Rastegar,
University of Manitoba, Canada

Reviewed by:
Moshe Szyf,
McGill University, Canada
Dennis Grayson,
University of Illinois at Chicago, United States

****Correspondence:***
Fushun Wang
13814541138@163.com

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leads to HPA-axis hyper-reactivity in adulthood, leading to monoaminergic dysfunction and, thus, major depression.

Indeed, adversity in early life will have long-term impact on the expressions of many genes, including the HPA axis and monoaminergic transporters or receptors, and monoamine oxidase (Chen et al., 2021). These systems could be affected by epigenetic changes, including methylation of cytosine in genomic DNA or mRNA modification at A, G, and C. Since methylation of cytosine levels influences gene expression and can be long-lasting, altered methylation of cytosine at specific sites or throughout the genome is hypothesized to influence mental and physical outcomes. For example, some early life events induce methylation of the glucocorticoid receptor gene (Nr3c1) and DNA methylation of MAO (monoamine oxidase) (Gu et al., 2016). These epigenetic mechanisms make cell-specific gene expression possible and allow for a genome to be programmed in multiple ways, resulting in a variety of stable gene expression profiles (Kronman et al., 2021). In all, both human and animal studies have shown that monoaminergic neurotransmitter and HPA axis function may be altered by abnormal epigenetic modifications, and these changes will affect the expression of certain proteins under specific stress conditions, and cause affective disorders (Chen et al., 2021). In this special issue, we are pleased to have received 18 total submissions with 10 of them accepted for publication after rigorous peer review. These papers focus on early life traumatic events, which are mediated by epigenetic alternations in affective disorders.

In the paper titled "Early Life Stress- and drug-induced epigenetic modifications of histones in the Ventral Tegmental Area," Shepard and Nugent provide a focused review on differential histone modifications within the ventral tegmental area (VTA), as well as their relevance to disease-based phenotypes, specifically focusing on epigenetic dysregulation of histones in the VTA associated with early life stress and drugs of abuse. They suggest that locus- and cell type-specific targeting of individual histone modifications at specific genes within the VTA presents novel therapeutic targets that can result in greater efficacy and better long-term health outcomes in susceptible individuals that are at increased risk for substance use and psychiatric disorders.

Wang et al. investigated the molecular mechanisms mediating the effects of early life stress on the development of psychiatric disorders in later life, including anxiety, depression as well as cognitive impairments. They evaluated the changes of mTOR signaling after maternal separation and chronic restraint stress model and analyzed mRNA levels and proteins expression of PSD95 and synaptophysin in the hippocampus. They also examined the activity of mTOR and S6. Their results indicate that maternally separated mice show decreased levels of both mRNA and protein for PSD95, synaptophsin in the hippocampus,as well as decreased phosphorylated mTOR and phosphorylated S6 in maternally separation mice vs. those non-exposed to maternal separation. They concluded that early maternal separation experience impaired synaptic plasticity and inhibited the mTOR signaling pathway, specifically via S6, and thus decreased synaptic plasticity via the inhibition of the mTOR pathway in the hippocampus, which may underlie vulnerability to mental disorders in adulthood. Their research paper is titled "Early-life stress alters synaptic plasticity and mTOR signaling: correlation with anxiety-like and cognition-related behavior."

In the review paper by Sogo et al., titled "Genetic and epigenetic consequence of early-life social stress on depression: role of serotonin associated genes," they examined the evidence for early-life social stress-induced epigenetic changes that modulate juvenile and adult social behavior (depression and anxiety). Their review has a particular emphasis on the interactions between early-life social stress and genetic variation of serotonin associate genes, including the serotonin transporter gene (5-HTT; also known as SLC6A4), which are key molecules involved in depression.

Su et al. studied strand-specific next-generation RNA sequencing, and reported in the research article "Gene transcript alterations in the spinal cord, anterior cingulate cortex, and amygdala in mice following peripheral nerve injury." They observed the changes in whole transcriptomes in the spinal cord, anterior cingulate cortex, and amygdala following unilateral fourth lumbar spinal nerve ligation. They found that the most significantly enriched biological processes amongst the upregulated mRNAs were involved in the immune system process, apoptotic process, defense response, inflammation response, and sensory perception of pain.

In the research article "Differentially expressed genes in the brain of aging mice with cognitive alteration and depression- and anxiety-like behaviors," Li, Su et al. examined differentially expressed genes and genes with differentially expressed isoforms in the anterior cingulate cortex, amygdala, and hippocampus throughout the lifespan of mice. The RNA sequencing analysis identified 634 and 1,078 differentially expressed genes in ACC, 453 and 1,015 differentially expressed genes in the amygdala, and 884 and 1,054 differentially expressed genes in hippocampus in the 12- and 24-months old mice, respectively. They found that all of these genes with differentially expressed isoforms were closely related to neuroinflammation. They concluded that these neuroinflammation-related genes are likely new targets in the management of memory/cognitive impairment and emotional disorders during aging.

Li, Fu et al. reviewed both clinical and preclinical studies and found that early life stress-induced epigenetic changes of the HPA axis, monoamine, neuropeptides in DNA methylation, histone modification, and RNA transcriptions associated with early life stress might be mediating depression later in life. Epigenetic alterations may add fuel to the fire in the process of depression by blunting the response to antidepressants in those with depression with a history of early life stress. However, they concluded that more research needed to be done to investigate the direct evidence for early life stress-induced epigenetic changes to contribute to the vulnerability of depression, summarized in their review paper, "Effect of early life stress on the epigenetic profiles in depression."

In the research paper "Long-term Effect of Post-traumatic Stress in Adolescence on Dendrite Development and H3K9me2/BDNF Expression in Male Rat Hippocampus and Prefrontal Cortex," Zhao et al. established a model of PTSD in adolescent rats using an inescapable footshock procedure. They

tested anxiety-like behaviors, social interaction behavior, and memory function with the open field test, elevated plus maze test, three-chamber sociability test, and Morris water maze test. They also examined neuronal development and H3K9me2/BDNF expression in the hippocampus and prefrontal cortex with Golgi staining, Western blotting, qRT-PCR analysis, and CHIP-qPCR analysis. Their results showed that the footshock procedure induced PTSD-like behaviors in rats, resulted in fewer dendrite branches and shorter dendrite length in CA1 of hippocampus and prefrontal cortex, increased H3K9me2 levels, and decreased BDNF expression in the hippocampus and prefrontal cortex.

Wei et al. suggested, in their research paper "Involvement of oxytocin receptor/Erk/MAPK signaling in the prefrontal cortex in early life stress-induced autism-like behaviors", that the infant period is a critical stage for the development of brain neuroplasticity. They found that juvenile rats subjected to daily 4-h neonatal maternal separation during postnatal day 1 to 20 exhibited autism-like behavioral deficits without impairments in learning and memory functions. Molecular studies showed that oxytocin receptors (OXTR) in the prefrontal cortex of neonatal maternal separation rats were evidently down-regulated when compared with control pups.

In a review paper, Gu et al. (2016) reviewed recent advances in the understanding of the structure and physiological function of monoamine oxidases (MAO), and they also briefly discussed other factors, including stress-induced changes, early life stress, perinatal depression relationship with other epigenetic changes, such as DNA methylation, and microRNA. The paper confirmed the conclusion that early life stress is a significant cause for major depressive disorder, which is one of the leading causes of human disabilities. The paper is titled "Early Life Stress Induced DNA Methylation of Monoamine Oxidases Leads to Depressive-like Behavior."

In conclusion, epigenetics studies in both animals and humans have given rise to new mechanisms of how early life stressors induce depression-related behaviors. Epigenetic mechanisms, which mediate the life-long effects of perinatal adversity, are potentially attractive targets for early detection, intervention, and prevention of mental health disorders.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Network Analysis of miRNA and mRNA Changes in the Prelimbic Cortex of Rats With Chronic Neuropathic Pain: Pointing to Inflammation

Guohong Cai[1†], Yuanyuan Zhu[1†], Yan Zhao[2†], Jing Chen[3], Chihua Guo[4], Feifei Wu[5], Jing Huang[1] and Shengxi Wu[1*]*

[1] Department of Neurobiology, School of Basic Medicine, Fourth Military Medical University, Xi'an, China, [2] Department of Gastroenterology, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China, [3] Department of Anatomy, School of Basic Medicine, Fourth Military Medical University, Xi'an, China, [4] Institute of Basic Medical Sciences, Xi'an Medical University, Xi'an, China, [5] Basic Medicine Teaching Experiment Center, School of Basic Medicine, Fourth Military Medical University, Xi'an, China

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Edited by:
Fushun Wang,
Nanjing University of Chinese Medicine, China

Reviewed by:
Jing Cao,
Zhengzhou University, China
Zang Weidong,
Zhengzhou University, China

***Correspondence:**
Jing Huang
jinghuang@fmmu.edu.cn
Shengxi Wu
shengxi@fmmu.edu.cn

[†] These authors have contributed equally to this work

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Neuropathic pain (NP) is a complex, chronic pain condition caused by injury or dysfunction affecting the somatosensory nervous system. This study aimed to identify crucial mRNAs and microRNAs (miRNAs) in the prelimbic cortex (PL) of NP rats. mRNA and miRNA microarrays were applied in the present study. The miRNA-mRNA regulatory network was constructed by using ingenuity pathway analysis (IPA). A total of 35 differentially expressed (DE) RNAs (24 miRNAs and 10 mRNAs) were identified in the spared nerve injury (SNI) group compared with the control group. The DE miRNA-mRNA network showed that IL-6 and tumor necrosis factor (TNF) were core components. Mir-30c-5p and mir-16-5p were the most connected miRNAs in the network. Interestingly, four mRNAs (Rnase 4, Egr2, Rexo4, and Klf2) with significantly increased expression were abundantly expressed in microglia, which was verified by the real-time quantitative polymerase chain reaction (qPCR). Furthermore, the expression of Rnase4 and Egr2 decreased in M1-polarized macrophages and increased in M2-polarized macrophages. In conclusion, we screened dozens of DE mRNAs and miRNAs in the PL of SNI rats. The core of the DE mRNA and miRNA network pointed to molecules associated with inflammation. Four mRNAs (Rnase4, Egr2, Rexo4, and Klf2) might be the potential markers of M2 polarization.

Keywords: neuropathic pain, prelimbic cortex, microRNA, mRNA, microglia, inflammation

INTRODUCTION

One-fifth of the world's population suffers from chronic pain (Reid et al., 2011), which is accompanied by many comorbidities, including mood disorders, deficits in cognition and memory, inattention and decreased motivation (Simons et al., 2014). Clinically, a large percentage of patients with chronic pain are accompanied with anxiety (Dersh et al., 2002). Similarly, patients with anxiety usually show more pain symptoms and cognitive impairment (Bair et al., 2003). Due to the

interactions between pain and anxiety, the treatment of chronic pain becomes more challenging. Moreover, the specific molecular mechanism involved in chronic pain-induced anxiety is not yet fully understood. Unfortunately, as of now, the specific mechanism of chronic pain-induced anxiety remains unclear.

In chronic pain, pain signals are transmitted to several brain regions, which are thought to be involved in the initiation of anxiety (Alba-Delgado et al., 2013). We previously showed the role of the anterior cingulate cortex (ACC) in affective responses to pain and related anxiety-like behavior (Guo et al., 2017). Functional imaging studies suggested that in addition to the ACC, the prefrontal cortex (PFC) was also involved in pain-related mood disorders (Seifert and Maihofner, 2009). The medial PFC (mPFC) has been shown to play an important role in pain-related perception and emotional processes (Hung et al., 2014). In a chronic pain animal model, Metz et al. (2009) reported the abnormalities in the function and morphology of the mPFC. The prelimbic cortex (PL), a subregion of the mPFC, might be a critical region in the pathological process of pain-induced anxiety (Wang et al., 2015). The pyramidal cells in different layers of the PL from neuropathic pain mice exhibited different excitability (Mitric et al., 2019). However, the identification of more molecular mechanisms is needed.

MicroRNAs (miRNAs) are noncoding genes that contain 19–25 nucleotides, which play a pivotal role in controlling biological processes by affecting messenger RNA (mRNA) stability and protein translation (Lopez-Gonzalez et al., 2017). There are a large number of reports of miRNA changes in the spinal cord of neuropathic pain models (Gong et al., 2015; Liu et al., 2018; Cao et al., 2019), whereas only a few studies have examined the mPFC (Li et al., 2019). We hypothesized that changes in miRNA and mRNA expression in the PL might influence behavioral changes in neuropathic pain and will become potential targets for pain control therapy. Based on this evidence, in this study, we sought to determine the key changes in miRNA and mRNA expression in PL that might influence neuropathic pain and related anxiety.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley (SD) rats (200–250 g, 6–7 weeks of age) were acquired from the Experimental Animal Center of Medical University. All experimental animals were housed in groups in a temperature- and humidity-controlled room with a 12:12 h light-dark cycle and water available ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH). Every effort has been made to minimize animal suffering and reduce the number of animals used.

Surgery

Spared nerve injury surgery was performed as described (Decosterd and Woolf, 2000). Briefly, under pentobarbital anesthesia, the tibial branches and sural common peroneal of the left sciatic nerve were exposed. Then, the tibial and common peroneal nerves were transected, while the sural nerve was kept intact. For the sham group, nerves were exposed but not transected.

Behavioral Testing

Mechanical Allodynia Test

Mechanical allodynia was measured by the paw withdrawal threshold (PWT) in response to a series of von Frey hairs ranging from 0.4 to 60 g. Rats were placed individually into Plexiglas chambers on an elevated wire-mesh floor, and the von Frey hairs were applied to the plantar surface of the hind paw with increasing forces until the rat withdrew. The lowest force that produced at least 3 withdrawal responses in five consecutive applications was defined as the PWT.

Open-Field Test

Rats were brought into the test room and allowed them to acclimate for at least half an hour before testing. The open-field (OF) arena was a black plastic box measuring 100 × 100 × 40 cm, and the test was performed under full-light conditions (1000 lux). Rats were placed in the center of the arena and their behavior was recorded for 5 min from above. After the test, rats were returned to their cages and the arena was wiped with ethanol. The automated analysis system (SMART 3.0, Panlab S.L.U.) was used to calculate the time spent in the center of the arena.

Lesions of the Prelimbic Cortex

The specific steps were mainly referring to the previous reference (Wang et al., 2015). On day 7 after surgery, the rats were anesthetized with pentobarbital, 300 mg per 100 g intraperitoneally, and positioned in a stereotaxic instrument. The injection of prelimbic cortex (PL) was according to the atlas of Paxinos and Watson (1997) (AP, +2.8, ML, ±0.5, DV, −4 mm). A 0.5 μl volume of a 0.1-M solution of quinolinic acid (QA), in phosphate buffered saline (0.1 mol/L, pH = 7.4) was administered in each side of PL in 1 min (0.5 μl/min). The rats in control group were injected with saline in the same way. After the surgery, the animal was returned to its home cage. Seven days after the surgery of lesion, OF and PWL were assessed. After the behavior tests, the location and effect of the injection were detected by immunofluorescence. The labeling of glial fibrillary acidic protein (GFAP) or neuronal nuclei protein (NeuN) was performed on free floating 40 μm thick transverse sections from rats perfused with 4% paraformaldehyde 7 days following injection. The sections were incubated with rabbit anti-GFAP (1:300, Dako) or anti-NeuN (1:500, Abcam) for 15 h at 4°C. Secondary antibody was donkey anti-rabbit Alexa Fluor 488 (1:1000, Jackson). Sections were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). Images were captured with an Olympus FV1000 confocal scanning laser microscope with appropriate laser scanning and filter for Alexa 488.

mRNA and miRNA Microarray Analysis

On day 14 after surgery, rats were anesthetized with pentobarbital, 300 mg per 100 g intraperitoneally, and were decapitated immediately. The brains were rapidly removed,

and the contralateral PL regions of the brains were dissected. Three samples of each group were tested for mRNA and miRNA microarray analysis. PL tissue RNA was isolated with the TRIzol reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Affymetrix GeneChip Rat Genome 230 Array 2.0 and GeneChip® Rat miRNA 4.0 Array (Affymetrix; Thermo Fisher Scientific, Inc.) were applied in the present study and all associated procedures were conducted by GeneChem Co., Ltd., (Shanghai, China), according to the standard operating procedure. All data were corrected by RMA method. Then, log2 transformation along with quantile normalization was applied subsequently. Expressional data were analyzed in R software using the Limma package to identify differentially expressed genes. The microarray data was deposited in the GEO repository (GSE145226, mRNA) and (GSE145199, miRNA).

Network Analysis and Visualization

The interactome of differentially expressed (DE) mRNAs and miRNAs was generated using the Ingenuity Pathway Analysis software (Ingenuity Systems[1]). Only experimentally observed microRNA-target interactions and those predicted with high confidence were used. IPA assigns "high confidence" to interactions involving a conserved or highly conserved miRNA as defined by TargetScan (Lewis et al., 2005; Garcia et al., 2011; Osterndorff-Kahanek et al., 2018). In addition, we limited the network data to genes related to anxiety, anxiety disorders and discomfort.

Cell Culture Treatments

The N9 cell line was purchased by ATCC (American Type Culture Collection). The N9 cells were plated on 6-well culture dishes at a density of $\sim 1 \times 10^5/cm^2$. The N9 cells were maintained in culture in DMEM, 10% heat-inactivated FBS, 1% penicillin & streptomycin Pen Strep (Gibco), and 1% glutamine (Sigma). First, the N9 cells were washed three times with PBS. Then, the N9 cells were incubated 24 h with LPS (25 ng/ml) (L4391, Sigma) for M1 polarization, and the cells were incubated 24 h with IL-4 (20 ng/ml) (SRP4137, Sigma) for M2 polarization.

[1]www.ingenuity.com

qRT-PCR Analysis

The N9 cells were lysed by TRIzol lysis buffer. Then, the total RNA from lysis buffer was isolated using a RNAprep Pure Cell kit (Tiangen) according to the manufacturer's instructions. The total RNA from PL tissues was isolated using a tissue total RNA extraction kit (Tiangen, DP431) according to the manufacturer's instructions. The specific experimental steps are as described in the previous article (Zhu et al., 2020). The mRNA was retrotranscribed into cDAN a total of 1 μg using a PrimeScriptTM RT reagent Kit (TaKaRa). Target cDNA levels were determined by RT-PCR (Thermo Fisher Scientific, Wilmington, MA, United States) using TB Green kit (TaKaRa) according to the manufacturer's instructions. The amplification assays were performed in 25 μL reaction mixtures containing 2 × TB Green Premix. PCR was performed using 2 μL of the cDNA solution, 12.5 μL of TB Green mix, 1 μL of each primer (10 μM), and 8.5 μL of ddwater. The PCR profile was 1 min at 95°C, 45 cycles of 5 s at 95°C, and 20 s at 60°C. The cDNA was normalized with GAPDH. The forward and reverse PCR primers were as follows:

TNFα: 5′-ACACCATGAGCACAGAAAGC-3′ and 5′- GCC ACAAGCAGGA ATGAGAAG-3′;

IL-1β: 5′- TCTCGCAGCAGCACATCAAC-3′ and 5′- ACCA GCAGGTTATCAT CATCATCC;

iNOS: 5′- CCCTTCAATGGTTGGTACATGG-3′ and 5′- ACA TTGATCTCCGTG ACAGCC-3′;

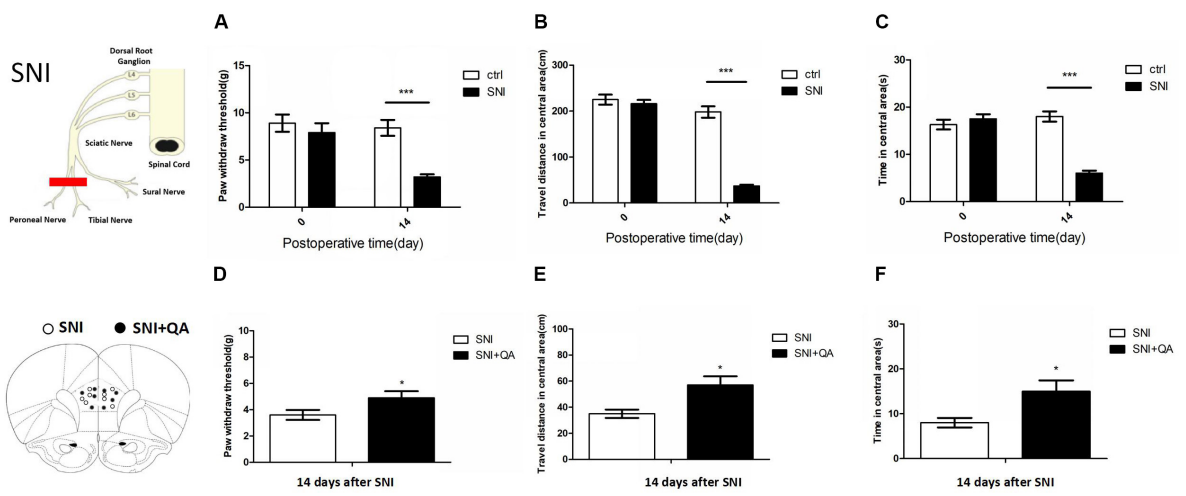

FIGURE 1 | The SNI model of neuropathic pain induces mechanical allodynia and enhances anxiety-like behaviors. **(A)** Fifty percent of mechanical thresholds of mechanical allodynia were determined using von Frey filaments before surgery (baseline) and 14 days after surgery. (n = 8 rats per group; $^{***}P < 0.001$, two-tailed t-test). **(B,C)** Anxiety-like behaviors assessed in an OF test (n = 8 rats per group; two-tailed t-test). $^{***}P < 0.001$. **(D–F)** Paw withdrawal threshold and open field of the SNI rats after bilateral lesion of the PL by quinolinic acid, respectively (n = 8 rats per group; $^{*}P < 0.05$, two-tailed t-test). The data are presented as mean ± s.e.m.

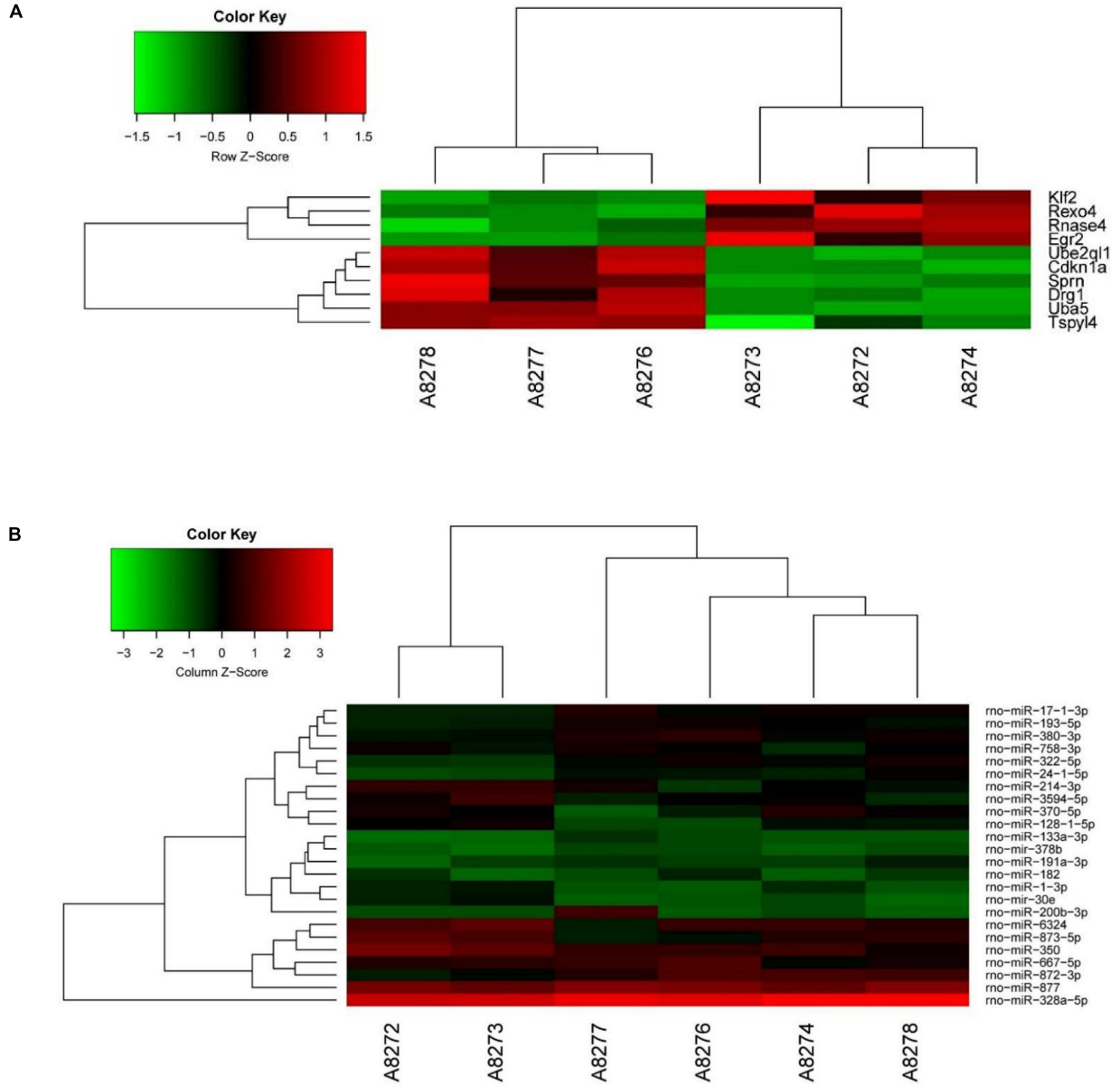

FIGURE 2 | The clustering map and network analysis of DE mRNAs and miRNAs. **(A)** There were 4 up-regulated mRNAs and 6 down-regulated mRNAs. **(B)** There were 25 DE miRNAs (10 up-regulation and 15 down-regulation).

IL-10: 5′-CTCTTACTGACTGGCATGAGGATC-3’ and 5’-AAGGAGTCGGTT AGCAGTATGTTG-3′;

Arg-1: 5′-CTCCAAGCCAAAGTCCTTAGAG-3′ and 5′- AG GAGCTGTCAT TAGGGACATC-3′;

Rnase4: 5′-CCAGTGCAAACGCTTCAACA-3′ and 5′-TGA CAACTCGCCT AGTGCTG-3′;

Egr2: 5′-CTCAGTGGTTTTATGCACCAGC-3′ and 5′-GAT GGGAGCGAAGC TACTCG-3′;

Rexo4: 5′-GCCTATCCGGAAGCTCGTTA-3′ and 5′-CTTG CAGCGCCT TCCAATTT-3′;

Klf2: 5′-CTCAGCGAGCCTATCTTGCC-3′ and 5′-CCAGTC CCATG GACAGGATG-3′.

Western Blot Analysis

The experimental process is as described in the previous study (Guo et al., 2017). PL tissues of SNI rats and control rats were collected and lysed in 100–300 ml of radio immunoprecipitation assay (RIPA) lysis buffer. The BCA protein assay (Pierce) was used to quantify the total protein samples (20–40 mg), and then the samples were resolved via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE) and transferred to PVDF membranes. The primary antibodies were as follows: mouse anti-β-actin (1:1,000, Cell Signaling Technology); rabbit anti-Rnase4 (1:1,000, Abcam); rabbit anti-Egr2 (1:1,000, Millipore). The immunoblots were incubated with the following

secondary antibodies: horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG, HRP-conjugated donkey anti-rabbit IgG (1:5,000, Invitrogen). The enhanced chemiluminescence (ECL) detection method (Advansta) was used to visualize all Western blots. Image J software (version 1.47) was used to quantify the scanned images.

Statistical Analyses

The data are expressed as the means ± SEM. Testing of statistical significance was performed using the unpaired *t*-test, two-sided *t*-test. Statistical significance was set to $p < 0.05$. *P* values were classified as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

RESULTS

The SNI Model Induces Mechanical Allodynia and Anxiety-Like Behaviors

Rats in SNI group showed decreasing mechanical thresholds in the von Frey test for 14 days, which indicated robust mechanical allodynia (**Figure 1A**). In addition, anxiety-related exploratory behavior of rats was assessed by OF test 14 days after surgery. Compared with the control group, SNI rats traveled shorter distances and spent significantly less time in the center area of OF test (**Figures 1B,C**). These data showed that SNI induced long-term persistent nociceptive sensitization and increased in anxiety-like behaviors.

According to a previous study, the PL was the specific subregion of mPFC that was responsible for pain and related anxiety (Wang et al., 2015). We bilaterally infused quinolinic acid (QA), a potent neurotoxic compound, into the PL to damage it (**Supplementary Figure S1**). After bilateral lesion of the PL, SNI rats exhibited increased paw withdraw threshold and increased travel distance and time in the center area of the OF (**Figures 1D–F**), suggesting the vital role of the PL in modulating pain sensation and related anxiety-like behaviors.

Differentially Expressed (DE) mRNAs and miRNAs

We analyzed DE mRNAs in the PL using a significance analysis of the GeneChip® Rat Genome 230 2.0 Array, following the criteria log2-fold-change >1.5 and $p < 0.05$. **Figure 2A** shows the clustering map of the DE mRNAs. The information on the upregulated and downregulated mRNAs in the SNI group compared with the control group on 14 days after SNI has been shown in **Table 1**. There were four mRNAs with upregulated expression and six mRNAs with downregulated expression in the SNI group compared to the control group.

We analyzed the DE miRNAs in the PL using significance analysis of the GeneChip® Rat miRNA 4.0 Array using the criterion of log2-fold-change >1.5. **Figure 2B** shows the clustering map of DE miRNAs. There were 24 DE miRNAs (9 upregulated and 15 downregulated) in the rat PL on 14 days in the SNI group compared to the control group. Information on the miRNAs with upregulated and downregulated expression in the SNI group has been shown in **Table 2**.

TABLE 1 | The top 10 upregulated and downregulated differentially expressed genes (DEGs).

Gene symbol	Gene_location	Fold change	*P*-value	Regulation
Rexo4	chr3:5849502–5850253	2.0487504	0.006235537	up
Klf2	chr16:17941333–17942639	1.8269434	0.013266017	up
Rnase4	chr15:27089667–27090445	2.1179023	0.001291831	up
Egr2	chr20:21883885–21888177	3.1384263	0.007462819	up
Tspyl4	chr20:38640484–38641266	−1.913118	0.006247621	down
Ube2ql1	chr17:3739696–3740380	−1.534941	0.001901304	down
Sprn	chr1:199953670–199953768	−1.5222503	0.004164148	down
Uba5	chr8:109108739–109115887	−1.9610097	$9.06281E-05$	down
Drg1	chr14:83857802–83860049	−1.6962591	0.008923944	down
Cdkn1a	chr20:7379385–7385634	−1.5424892	0.00128275	down

TABLE 2 | The detail information of the up-regulated and down-regulated miRNAs.

miRNA	Alignments	Fold change	Regulation
rno-miR-873-5p	5:53454961–53454981 (–)	1.842379163	up
rno-miR-370-5p	6:134210206–134210229 (+)	1.670982935	up
rno-mir-30e	5:141365115–141365206 (–)	1.668093704	up
rno-miR-3594-5p	10:109476519–109476539 (–)	1.610032711	up
rno-miR-128-1-5p	13:40907589–40907609 (+)	1.600926048	up
rno-miR-1-3p	18:2191683–2191704 (–)	1.595218818	up
rno-miR-6324	11:36339331–36339353 (–)	1.546903643	up
rno-miR-214-3p	13:77916255–77916275 (+)	1.514865807	up
rno-miR-350	13:92545090–92545113 (–)	1.513648268	up
rno-miR-24-1-5p	17:7351012–7351033 (–)	2.109342448	down
rno-miR-322-5p	X:140000212–140000233 (–)	1.979034368	down
rno-miR-872-3p	5:114979254–114979275 (+)	1.952591289	down
rno-miR-380-3p	6:134391034–134391054 (+)	1.87513786	down
rno-miR-200b-3p	5:172899014–172899036 (–)	1.744825387	down
rno-miR-17-1-3p	15:99853785–99853806 (+)	1.663682346	down
rno-miR-191a-3p	8:113614416–113614437 (+)	1.662845351	down
rno-miR-667-5p	6:134400514–134400537 (+)	1.640664214	down
rno-miR-758-3p	6:134392042–134392063 (+)	1.614745351	down
rno-miR-133a-3p	18:2189230–2189251 (–)	1.612216322	down
rno-miR-193-5p	10:65901067–65901088 (+)	1.590683342	down
rno-miR-877	20:2958184–2958203 (+)	1.563840454	down
rno-mir-378b	5:5156348–5156431 (–)	1.560021234	down
rno-miR-182	4:57071298–57071322 (–)	1.534569869	down
rno-miR-328a-5p	19:35122612–35122630 (–)	1.527511597	down

Functional Prediction of DE miRNAs in SNI

To determine the functional roles of the miRNAs and their target genes in biological pathways, we performed gene ontology (GO)

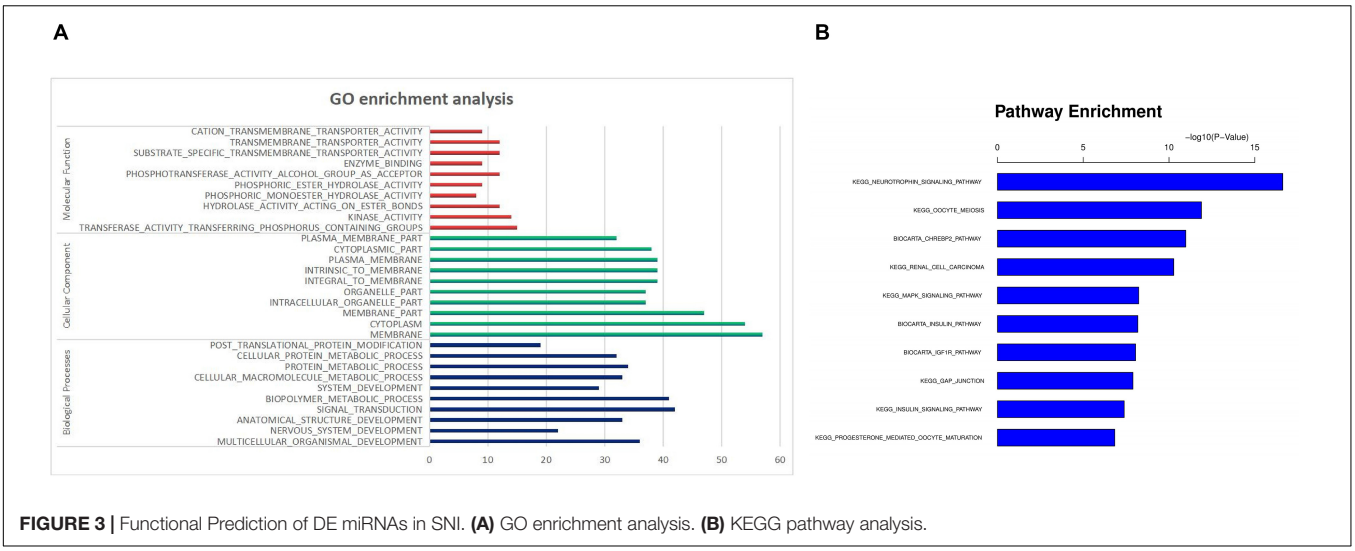

FIGURE 3 | Functional Prediction of DE miRNAs in SNI. **(A)** GO enrichment analysis. **(B)** KEGG pathway analysis.

TABLE 3 | KEGG Pathway analysis of DE miRNAs.

Gene set name	Gene number	*p*-Value	Gene name
KEGG_NEUROTROPHIN_SIGNALING_PATHWAY	17	2.36E-17	CRKL,IRAK2,BDNF,MAP2K1,NTF3,YWHAZ,ARHGDIA,YWHAG,IRS1, JUN,PIK3R2,YWHAH,YWHAE,RAF1,YWHAQ,MAPK3,YWHAB
KEGG_OOCYTE_MEIOSIS	13	1.29E-12	PPP2CA,YWHAG,PPP3R1,MAP2K1,BTRC,YWHAH,YWHAE,MAPK3, YWHAB,YWHAZ,YWHAQ,ADCY3,PPP2R1A
BIOCARTA_CHREBP2_PATHWAY	9	1.06E-11	PPP2R1A,PRKAR2A,YWHAZ,YWHAQ,YWHAB,YWHAE,YWHAH, YWHAG,PPP2CA
KEGG_RENAL_CELL_CARCINOMA	10	5.31E-11	VEGFA,EGLN3,RAF1,ETS1,MAPK3,PIK3R2,JUN,MAP2K1,CRKL,TCEB1
KEGG_MAPK_SIGNALING_PATHWAY	14	5.84E-09	PPM1B,PTPRR,RAF1,MAPK3,CACNB2,DUSP1,JUN,MAP2K1, CACNA1C,NTF3,CRKL,BDNF,MAP3K12,PPP3R1
BIOCARTA_INSULIN_PATHWAY	6	6.6E-09	RAF1,MAPK3,IRS1,JUN,MAP2K1,INSR
BIOCARTA_IGF1R_PATHWAY	6	8.88E-09	IRS1,MAP2K1,PRKAR2A,YWHAH,RAF1,MAPK3
KEGG_GAP_JUNCTION	9	1.26E-08	MAPK3,RAF1,LPAR1,TUBB3,GNAI2,ADCY3,TUBA1A,GNAI3,MAP2K1
KEGG_INSULIN_SIGNALING_PATHWAY	10	0.000000041	PRKAR2A,INSR,RAF1,MAPK3,PIK3R2,IRS1,MAP2K1,RPS6KB1,CRKL,CBLB
KEGG_PROGESTERONE_MEDIATED_OOCYTE_ MATURATION	8	0.000000145	GNAI2,ADCY3,RAF1,MAPK3,PIK3R2,MAP2K1,CDC25A,GNAI3

(Young et al., 2010) and KEGG pathway enrichment analysis for biological functions.

In the GO enrichment analysis, a total of 90 GO terms were significantly enriched. Thirty GO terms were mainly related to biological processes, such as multicellular organismal development, nervous system development and anatomical structure development. Thirty GO terms were related to cellular components, such as membrane, cytoplasm and intracellular organelle. And thirty GO terms were mainly related to molecular function, such as transferase activity, kinase activity, hydrolase activity and phosphoric ester hydrolase activity. The significantly enriched GO terms are shown in **Figure 3A** and **Supplementary Table S1**.

In the KEGG pathways analysis, approximately 10 significant pathways were detected and are shown in **Figure 3B** and **Table 3**. Notably, most genes were associated with neurotrophin signaling, oocyte meiosis, biocarta chrebp2, renal cell carcinoma and the mitogen activated kinase-like protein (MAPK) signaling pathway followed by the biocarta insulin, biocarta insulin like growth factor 1 receptor (IGF1R), gap junction, insulin signaling and progesterone mediated oocyte maturation pathways.

Network Analysis of DE mRNAs and miRNAs

To examine the molecular mechanism involved in NP-related anxiety pathogenesis, we performed a regulatory network analysis of the mRNAs and miRNAs involved in SNI pathogenesis. miRNAs were at the center, mRNAs were the target, and the regulation network was built by ingenuity pathway analysis (IPA) (**Figure 4**). Only experimentally observed microRNA-target interactions and those predicted with high confidence were used. These results illustrated the regulatory relationship between mRNA and miRNA in the mechanism of NP-related anxiety. The network showed that interleukin 6 (IL-6) and tumor necrosis factor (TNF) were most associated with the DE mRNAs and miRNAs, which suggested that inflammatory factors might play an important role in chronic pain with anxiety. Mir-30c-5p and mir-16-5p were the most connected miRNAs in the

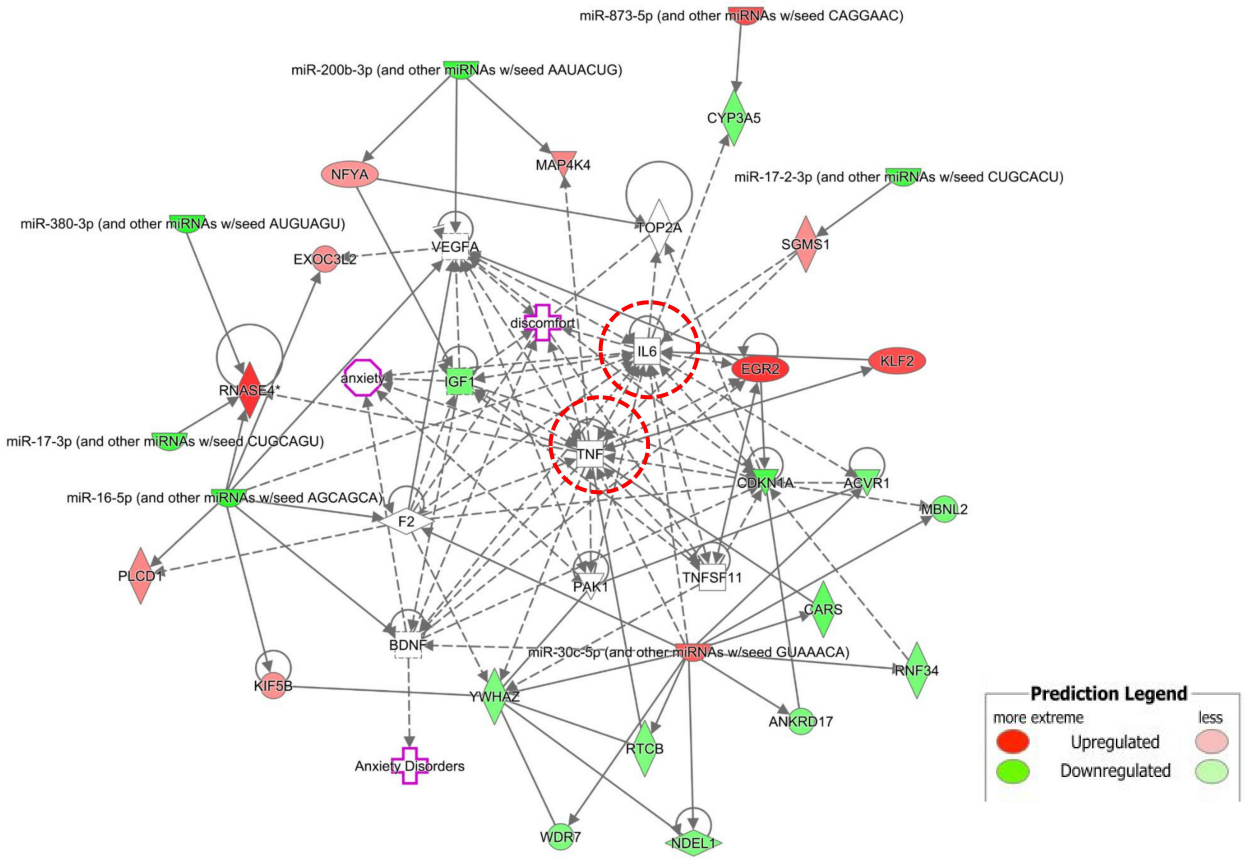

FIGURE 4 | The regulation network was built by Ingenuity Pathway Analysis (IPA). IL-6 and TNF were most associated mRNAs. Mir-30c-5p and mir-16-5p were most connected miRNAs. There were some other core genes in the network, including BDNF, CDKN1a, Egr2 and VEGFA.

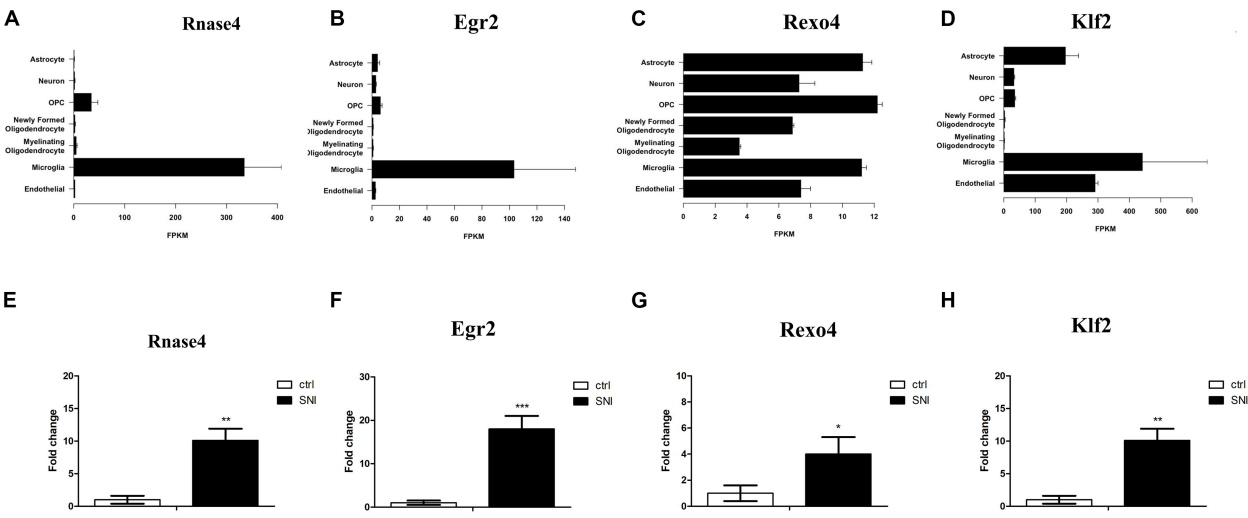

FIGURE 5 | Real-Time Quantitative Polymerase Chain Reaction (qPCR) Validation of key DE mRNAs Expression. **(A–D)** Based on an RNA-Seq transcriptome and splicing database of the cerebral cortex, four genes (Rnase4, Egr2, Rexo4, and Klf2) with significantly increased expression were found abundantly expressed in microglia. The expression level estimation was reported as fragments per kilobase of transcript sequence per million mapped fragments (FPKM) value together with confidence intervals for each sample. **(E–H)** These four mRNAs were analyzed by qPCR, and results were consistent with the results obtained from microarray analysis. $n = 3$ per group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed t-test. The data are presented as mean ± s.e.m.

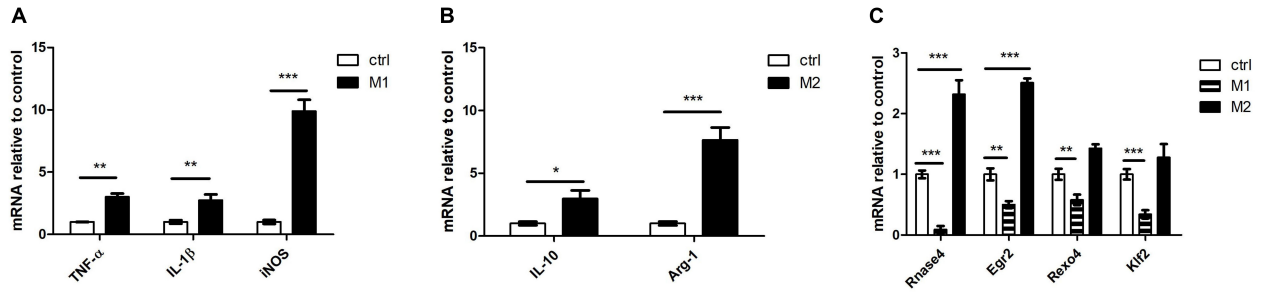

FIGURE 6 | The four DE mRNAs expression change in microglial M1 and M2 polarization. **(A)** qPCR for M1 polarization (TNF-α, IL-1β and iNOS) markers in LPS-stimulated N9 cells. **(B)** qPCR for M2 polarization (IL-10 and Arg-1) markers in IL-4-stimulated N9 cells. **(C)** Rnase4, Egr2, Rexo4, and Klf2 were analyzed by qPCR in microglial M1 and M2 polarization. $n = 3$ per group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed t-test. The data are presented as mean ± s.e.m.

network. There were some other core genes in the network, including brain derived neurotrophic factor (BDNF), cyclin dependent kinase inhibitor 1A (CDKN1a), early growth response 2 (Egr2) and vascular endothelial growth factor A (VEGFA). Indeed, the regulatory role of mRNAs and miRNAs in anxiety pathogenesis is very complicated, so an in-depth study is still needed in the future.

qRT-PCR and Western Blot Validation of mRNA Expression

Based on an RNA-Seq transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex (Zhang et al., 2014), we searched for the expression of the DE mRNAs in different types of cells. Interestingly, we found that four genes with significantly increased expression were abundantly expressed in microglia, including ribonuclease A family member 4 (Rnase4), Egr2, REX4 homolog, 3′-5′ exonuclease (Rexo4) and Kruppel like factor 2 (Klf2) (**Figures 5A–D**). These four mRNAs were analyzed by qPCR to validate the reliability of the microarray results and to provide the research a basis for further study. The expression of all of the validated mRNAs was consistent with the microarray results (**Figures 5E–H**). Western blot was then performed to verify the two most significant genes Rnase4 and Egr2 of the four differential genes. In the PL of the SNI model 14 days after surgery, the protein levels of Rnase4 and Egr2 were significantly reduced when compared with control group (**Supplementary Figure S2**).

The Four DE mRNAs Changed Significantly in Microglial M1 and M2 Polarization

Microglia can be polarized toward an M1 or M2 phenotype depending on the local microenvironment (Colton, 2009). We chose LPS stimulation to induce microglial M1 polarization and chose IL-4 stimulation to induce microglial M2 polarization in N9 cells. We used TNF-α, IL-1β and inducible nitric oxide synthase (iNOS) as M1 markers and used IL-10 and arginase 1 (Arg-1) as M2 markers. There was a significant increase in TNF-α, IL-1β and iNOS expression following LPS stimulation (**Figure 6A**). The expression of IL-10 and Arg-1 was significantly increased following IL-4 stimulation (**Figure 6B**). Furthermore, we detected four DE mRNAs (Rnase4, Egr2, Rexo4 and Klf2) between M1- and M2-polarized macrophages. Our results showed that the expression of all four genes (Rnase4, Egr2, Rexo4 and Klf2) was significantly decreased in M1 polarization conditions and that Rnase4 and Egr2 expression was significantly increased in M2 polarization conditions (**Figure 6C**).

DISCUSSION

To our knowledge, this is the first study to screen DE mRNAs and miRNAs in the PL of SNI rats. The DE mRNA-miRNA network showed that IL-6 and TNF were in the central position, and mir-30c-5p and mir-16-5p were the most connected miRNAs in the network. Four genes (Rnase4, Egr2, Rexo4 and Klf2) with significantly increased expression were abundantly expressed in microglia. Furthermore, Rnase 4 and Egr2 expression was decreased in M1-polarized macrophages and increased in M2-polarized macrophages, suggesting their potential role in inhibiting inflammation.

In the healthy brain and spinal cord, microglia represent approximately 10% of CNS cells, which were once virtually ignored. By applying new technologies, more information about the roles of microglia has been obtained, which is growing at an ever-accelerating rate (Salter and Stevens, 2017). Research from Sorge et al. (2015) showed that pain depends on microglial signaling only in males. In this research, we used male SD rats and the details of this signaling mechanism remain to be determined.

Increasing studies have focused on the microglia in the peripheral NP (Beggs et al., 2012; Sorge et al., 2012). It was suggested that in spinal microglia, the activation of P2X4 receptors (P2X4Rs) led to a release of BDNF that subsequently acted on neurons to suppress inhibition, which eventually caused pain hypersensitivity (Masuda et al., 2016). Moreover, in 2016, the study by Li et al. (2016) provided the first evidence that spinal and brain microglia/macrophages had various functions in chronic neuropathic pain. Microglia regulated the function of neurons and synaptic plasticity by releasing inflammatory cytokines and molecules, such as interleukins or TNF (Salter and Stevens, 2017). In the current study, IL-6 and TNF were in the central positions of

the DE mRNA-miRNA network, which indicated that microglia were activated in the PL of NP rats.

Microglia have multiple activation phenotypes with different molecular phenotypes and functions that depend on their local microenvironment (Colton, 2009). For example, the M1 phenotype, with high levels of proinflammatory cytokines and oxidative metabolites, can be induced by lipopolysaccharide (LPS) or interferon gamma (IFNγ) (Lynch, 2009). The M1 phenotype is regard as a neurotoxic phenotype due to it may cause tissue destruction or injury. Conversely, IL-4 or IL-10 (anti-inflammatory cytokines) promotes the M2 phenotype, which plays a key role in repairing processes and suppressing destructive immune responses (Ponomarev et al., 2007; Colton, 2009).

Imbalanced microglial polarization to the M1 phenotype may lead to the development of pain, while shift of polarization of microglia to the M2 phenotype may contribute to the relief of pain (Pannell et al., 2016; Huo et al., 2018). Based on an RNA-Seq transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex (Zhang et al., 2014), we found that 4 DE mRNAs (Rnase4, Egr2, Rexo4 and Klf2) with significantly increased expression were abundantly expressed in microglia. Furthermore, the expression of all four genes was significantly decreased in M1-polarized cells, and Rnase4 and Egr2 expression was significantly increased in M2-polarized cells, suggesting that they potentially play an anti-inflammatory role.

In previous research, Egr2 expression was shown to be associated with M2 macrophage polarization (Veremeyko et al., 2018). Moreover, Egr2 labeled more M2 macrophages than the common M2 macrophage marker Arg-1 (Jablonski et al., 2015). Klf2, a nuclear transcription factor known to inhibit inflammation in endothelial cells and monocytes, was also involved in M2 macrophages (van Tits et al., 2011). The downregulation of Klf2 expression in M2 macrophages could lead to a significant increase in macrophage cationic peptide 1(MCP-1) secretion induced by LPS (van Tits et al., 2011). Our results showed that Egr2 and Klf2 expression was significantly decreased in M1-polarized macrophages and Egr2 expression was significantly increased in M2-polarized macrophages, which added new evidence for their role in M2 polarization.

Previous study showed that the expression of microglia M1 markers increases in PFC of rats on day 14 after SNI (Xu et al., 2017). Consistent with their results, Western blot results in current study showed the protein levels of Rnase4 and Egr2 were significantly reduced. However, Rnase4 and Egr2 mRNA were increased in PL of rats on day 14 after SNI. The polarization states of M1 and M2 of PFC may change at different times after SNI surgery (Li et al., 2016). In the PFC of SNI rats, the repairing effect of Rnase4 and Egr2 may start with an increase of mRNA expression before an increase of protein level. On day 14 after SNI, the increase in protein levels had not yet occurred. Chronic pain may also affect mRNA translation and protein modification processes, resulting in inconsistent changes in protein levels and mRNA. Subsequent studies need to use the SNI model for a longer time to verify whether the protein levels of Rnase4 and Egr2 increase during the recovery period. In the present study, we did not verify the role of differential genes in chronic pain or anxiety one by one, which we will continue to complete. In addition, the exact reasons of the differences in protein and gene expression (Rnase4 and Egr2) need to be verified by future studies.

Xu et al. (2017) showed that SNI increased CD68 and iNOS (M1 markers) expression significantly but failed to affect CD206, IL-4 or Arg-1 (M2 markers) expression in the prefrontal cortex. In fact, the negative M2 polarization result might be related to the choice of markers. Our results and previous literature suggested that M2 polarization might have other important markers. In addition, M2 polarization markers might be various in different regions (Tan et al., 2020).

In the DE mRNA-miRNA network, mir-30c-5p and mir-16-5p were the most connected miRNAs. Many previous studies have suggested that they were associated with M2 polarization (anti-inflammatory). Yang et al. (2020) showed that the M2 polarization of tumor-associated macrophages could be promoted by long noncoding RNA (lncRNA) RP11-361F15.2 through miR-30c-5p. A miR-30c-5p agomir might contribute to the transformation of M1 macrophages to M2 macrophages, resulting in changes in inflammatory cytokine levels (Zhang et al., 2019). A recent study showed that miR-16-5p mimics significantly decreased the mRNA expression of IL-1β, IL-6, and TNF-α under LPS stimulation conditions, which indicated that miR-16-5p might be a critical factor involved in the anti-inflammatory effects (Yamada et al., 2020). In another study, lncRNA small nucleolar RNA host gene 1 (SNHG1) reduced inflammation by activating the miR-16-5p-mediated p38 MAPK and the nuclear factor-κB (NF-κB) signaling pathways (Lei et al., 2019). However, the anti-inflammatory function of mir-30c-5p and mir-16-5p in the PL of chronic pain model still needs more research.

In conclusion, we screened dozens of DE mRNAs and miRNAs in the PL of SNI rats. The core of the DE mRNA and miRNA network pointed to molecules associated with inflammation (IL-6, TNF, miR-30c-5p and miR-16-5p). Four mRNAs (Rnase4, Egr2, Rexo4 and Klf2) with significantly increased expression were abundantly expressed in microglia and may be potential markers of M2 polarization. The mechanisms of these inflammation-related molecules in chronic pain need further study.

DATA AVAILABILITY STATEMENT

The microarray data was deposited in the GEO repository (GSE145226, mRNA and GSE145199, miRNA).

ETHICS STATEMENT

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Fourth Military Medical University and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH). Every effort has been made to minimize animal suffering and reduce the number of animals used.

AUTHOR CONTRIBUTIONS

SW and JH conceived and designed the experiments. GC, YuZ, YaZ, JC, CG, and FW performed the experiments. GC, YuZ, YaZ, and CG interpreted the data and prepared the figures. SW, JH, and GC wrote and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00612/full#supplementary-material

FIGURE S1 | The neuronal loss caused by injection of quinolinic acid into the PL. **(A)** Injection area of quinolinic acid (QA) in the PL (DAPI counterstaining). **(B)** High magnification images showing the immunostaining of glial fibrillary acidic protein (GFAP, green) around the QA injection site in the PL. **(C)** The immunostaining of NeuN (green) around the QA injection site in the PL (adjacent section of A). Bregma 3.80 mm. Scale bar: A, 500 μm; B and C 50 μm.

FIGURE S2 | Validation of Rnase4 and Egr2 protein Expression. **(A)** Western blot analysis of Rnase4 and Egr2 in the PL of SNI rats 14 days after surgery. **(B,C)** Rnase4 and Egr2 protein levels were reduced in SNI group. $n = 3$ per group; $^{*}P < 0.05$, $^{**}P < 0.01$, two-tailed t-test. The data are presented as mean ± s.e.m.

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Long-Term Effect of Post-traumatic Stress in Adolescence on Dendrite Development and H3K9me2/BDNF Expression in Male Rat Hippocampus and Prefrontal Cortex

*Mingyue Zhao, Wei Wang, Zhijun Jiang, Zemeng Zhu, Dexiang Liu and Fang Pan**

Department of Medical Psychology and Medical Ethics, School of Basic Medicine Sciences, Cheeloo College of Medicine, Shandong University, Jinan, China

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Fushun Wang,
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Jung Goo Lee,
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Peking University, China

***Correspondence:**
Fang Pan
panfang@sdu.edu.cn

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Exposure to a harsh environment in early life increases in the risk of post-traumatic stress disorder (PTSD) of an individual. Brain derived neurotrophic factor (BDNF) plays an important role in neurodevelopment in developmental stages. Both chronic and traumatic stresses induce a decrease in the level of BDNF and reduce neural plasticity, which is linked to the pathogenesis of PTSD. Also, studies have shown that stress alters the epigenetic marker H3K9me2, which can bind to the promoter region of the *Bdnf* gene and reduce BDNF protein level. However, the long-term effects of traumatic stress during adolescence on H3K9me2, BDNF expression and dendrite development are not well-known. The present study established a model of PTSD in adolescent rats using an inescapable foot shock (IFS) procedure. Anxiety-like behaviors, social interaction behavior and memory function were assessed by the open field test, elevated plus maze test, three-chamber sociability test and Morris water maze test. In addition, neuronal development and H3K9me2/BDNF expression in hippocampus (HIP) and prefrontal cortex (PFC) were evaluated by Golgi staining, western blotting, qRT-PCR analysis and CHIP-qPCR analysis. Additionally, the Unc0642, a small molecule inhibitor of histone methyltransferase (EHMT2) was used for intervention. The results showed that the IFS procedure induced the PTSD-like behaviors in rats, resulted in fewer dendrite branches and shorter dendrite length in CA1 of HIP and PFC, increased H3K9me2 level and decreased BDNF expression in HIP and PFC. Also, although all the changes can persist to adulthood, Unc0642 administration relieved most of alterations. Our study suggests that traumatic stress in adolescence leads to immediate and long-term mental disorders, neuronal morphological changes, lower BDNF level and increased H3K9me2 level in the HIP and PFC, indicating that H3K9me2/BDNF dysfunction plays a key role in pathogenesis of PTSD.

Keywords: PTSD, BDNF, H3K9me2, neurodevelopment, methylation, epigenetic

INTRODUCTION

Exposure to a harsh environment has a negative impact on brain structure that may lead to impairment of cognitive and emotional function in humans (Wang R. et al., 2018; Xu et al., 2019). Also, experiencing stress in early childhood may easily affect neuronal structure and function, thereby leading to substance abuse, anxiety, depression and even learning function and social communication impairments (Salzmann-Erikson and Hicdurmaz, 2017; Cowan et al., 2018; Postel et al., 2019; Ohta et al., 2020). In general, serious and life threatening injury events induce post-traumatic stress disorder (PTSD) which leads to long-term mental health problems, such as anxiety, depression and suicide (Friedman et al., 2011). The clinical symptoms of PTSD include recurrent and intrusive traumatic memories, avoidance of traumatic event-related stimuli, cognitive impairment, negative emotions, hyper-aroused state, hyper-vigilance, clinical distress, and social impairment (Richter-Levin et al., 2019). The pathogenesis of PTSD can involve many aspects, such as genetic factors, neurological, neuroendocrine and environmental factors (Pitman et al., 2012). However, dysregulation of dendrite development and synaptogenesis has been confirmed as a key factor in the etiology of PTSD (Heun-Johnson and Levitt, 2018).

Brain derived neurotrophic factor (BDNF) is a protein synthesized in the brain and widely distributed in the central and peripheral nervous systems. BDNF is considered to play an important role in neuronal differentiation, growth and development. In addition, BDNF has biological effects, such as preventing neuronal damage and death, improving neuronal pathological state, and promoting neuron regeneration (Lima Giacobbo et al., 2019; Voisey et al., 2019; Wen et al., 2020). An earlier study has shown that reduced expression levels of BDNF affect neuronal development and synaptic plasticity (Wang et al., 2015). In particularly, traumatic stress can significantly reduce the expression of BNDF in hippocampus (HIP) (Shafia et al., 2017; Alzoubi et al., 2019), impair synaptic plasticity in the prefrontal cortex (PFC) which are associated with susceptibility to trauma-related symptoms (Lguensat et al., 2019; Li et al., 2019; Zhang et al., 2019).

Many studies have shown that the expression of BDNF is involved in neuronal development and the epigenetic marker of H3K9me2/3 has a negative regulatory effect on transcription level of *Bdnf* gene (Walker et al., 2013; Kyzar et al., 2017). Recent studies have shown that H3K9me2 and its degree of methylation participate in regulating the occurrence of depression and anxiety symptoms in early maternally-separated separation young mice (Xu et al., 2018). More importantly, some studies suggest that stress-induced H3K9me2/3 undergoes irreversible changes, which have a regulatory effect on the expression of the *Bdnf* gene (Wu et al., 2019; Li et al., 2020). For example, continuous chronic exposure to morphine can trigger the methylation modification of H3K27 in the ventral segmental nucleus, which affects the transcriptional downregulation of *Bdnf* promoter sequences (Koo et al., 2015). Also, social isolation and chronic unpredictable stress induce the acetylation of H3K9 and H4K12 in their corresponding brain regions, which can reduce the expression level of BDNF and impair memory function in rats (Viana Borges et al., 2019). Such studies indicate that stress alters H3K9me2/3/BDNF signaling, which is involved in affective disorder and memory impairment. However, the role of traumatic stress in adolescence on dendrite development and H3K9me2/BDNF expression has not been elucidated. This study established a traumatic stress model in adolescent rats. In addition, Unc0642, a small molecule inhibitor of histone methyltransferase (EHMT2), was used to reduce the protein expression level of H3K9me2 during the stress procedure (Xiong et al., 2017; Wang et al., 2019). We hypothesized that traumatic stress in adolescence might induce behavioral dysfunction, dysregulation of neuronal development and maturation and changes in H3K9me2/3/BDNF expression in HIP and PFC, and those effects might persist to adulthood.

MATERIALS AND METHODS

Animals and Drug Treatment

A total of 72, 21-day old male Wistar rats weighing 60–80 g were purchased from the Animal Center of Shandong University (Jinan, Shandong, China). Rats (5 per cage) were housed in the Animal Center, in a controlled environment (12 h day/night cycle, 23 ± 2°C) and with *ad libitum* access to food and water. The experimental procedures conformed to the guidelines of the Animal Ethics Committee of Shandong University. Unc0642, a small molecule inhibitor of EHMT2 (G9a), can reduce the protein expression level of H3K9me2. Un0642 was dissolved in dimethyl sulfoxide (DMSO), polyethylene glycol (PEG) 300 and double-distilled water (DDW). The injection (i.p) (2.5 mg/kg/d) was administered 30 min after each shock (Berkel et al., 2019; Griñán-Ferré et al., 2019; Wang et al., 2019).

Experimental Design

After 7 days of adaptive feeding, the 72 male Wistar rats were randomly divided into three groups (24/group) according to their body weight, namely the control group, PTSD group and PTSD+Unc0642 group. Rats in the PTSD group received inescapable foot shock (IFS) for 6 days, while rats in the PTSD+Unc0642 group received both IFS and an intraperitoneal injection of Unc0642, for 6 and 10 days, respectively. Rats in control group and PTSD group were received intraperitoneal injection of saline for 10 days for balancing the operation error. After modeling, rats in each of the three groups were randomly divided into adolescent groups and adult groups. Then, rats in the adolescent groups were subjected to behavioral tests. For the adolescent rat groups, rats were sacrificed on the next day after the behavioral tests and brain tissues were collected for subsequent analysis. The rats in the adult groups were raised to 63 days of age and then subjected to the behavioral tests (see the **Figure 1**). Therefore, there are 6 groups in this study, namely the adolescent control group (AdoC, n = 12), the adolescent PTSD group (AdoP, n = 12), the adolescent Unc0642 medicated group (AdoP+U, n = 12), the adult control group (AduC, n = 12), the adult PTSD group (AduP, n = 12), and the adult Unc0642 medicated group (AduP+U, n = 12).

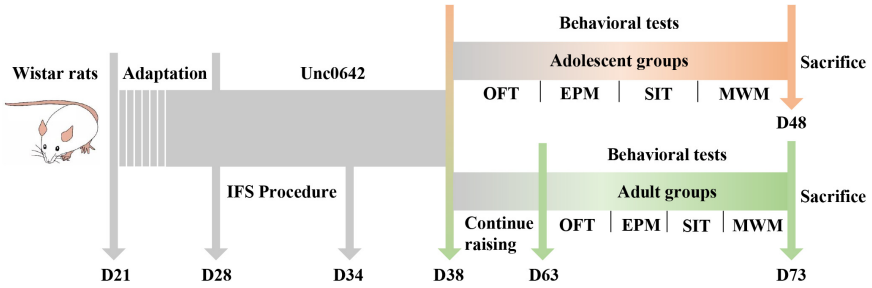

FIGURE 1 | Experimental flow chart.

The Inescapable Foot Shock (IFS) Procedure

The IFS procedure was used to establish a PTSD animal model (Ju et al., 2017). Rats were placed in an electric shock closed box, and the feet of the rats were subjected to consecutive unavoidable shocks for 18 times in a semi-random manner. Shock time interval was random, and the interval time was 30–120 s. IFS was performed twice a day, the interval was not less than 4 h. As a precaution to eliminate error interference, the rats in the control group were treated in a discharge box only protected from light but without electric shock.

Behavioral Tests

The exploration of new environment was evaluated by the open field test (OFT), the anxiety-like behavior was assessed by the elevated plus maze (EPM) test, the ability to explore novel environment and strange rats was evaluated using the social interaction test (SIT), and the spatial exploration and spatial memory capabilities were tested by the Morris water maze (MWM) test.

The Open Field Test

The open field experiment was performed to determine the autonomous movement and behavioral exploration ability of rats in a novel environment (Prut and Belzung, 2003). The rats were placed in the center of the experimental box, then the number of times of crossing, time spent in the central squares and the number of rearings of the rats were recorded for 5 min using the Smart software (SMART 2.5, Panlab). The test box was wiped with alcohol between tests in order to eliminate any residual olfactory cues left by the previously tested rat.

The Elevated Plus Maze Test

The elevated plus maze experiment was conducted to assess anxiety in rats (Walf and Frye, 2007). The elevated plus maze has two open arms and two closed arms that above 50 cm from the ground. The rats were placed at an elevated cross with their heads facing the open arms, and indexes of the number of times the rat entered into the open and closed arms and the time the rat in the open and closed arms in 5 min were recorded using the Smart software.

Three Chamber Sociability Test

Three social experiment chambers were used to test the ability to explore a novel environment and strange rats (social interaction behaviors) (Kaidanovich-Beilin et al., 2011). The experimental rectangular chamber with a top length of 120 cm, width of 60 cm, and height of 40 cm was divided into three parts of equal volume by a partition. Each partition has a small door of 20 × 10 cm. Rats were allowed to freely enter the three sections (left, center, right). Each of the left and right parts had a cage holding a strange rat. Before the experiment, rats were allowed to adapt to the experimental environment. The social experiment consisted of three phases, namely, the adaptation phase, the first phase and the second phase. In the 5 min adaptation phase, the rats were placed in the central part of the social box and the two doors were open, so that the rats could move freely to explore the social box. The first 5 min stage, the strange rat 1 is placed in either the left or right imprisonment cage, and the experimental rat is still placed in the central part of the social chamber with the two doors opened, so that the rat could freely shuttle through the two doors to explore the social chamber. The imprisonment cages that have been contacted during the adaptation phase, therefore, the number of times and time of exploring strange rat 1 represent the ability of experimental rats to explore novelty. In the second 5 min stage, the strange rat 2 is placed in the cage opposite to where the strange rat 1 was placed, and the experimental rat is still placed in the central part of the social chamber with the two doors opened, so that the rats can freely shuttle through the two doors to explore the social chamber. Compared to the strange rat 1 (the familiar rat at this stage) that has been contacted in the first 5 min stage, exploring the strange rat 2 showed the ability to explore novelty. The number of times and time that the experimental rat shuttled the left and right door are recorded, as well as the number of times and time of contact with the empty cage and the strange rat 1 and 2 in the first and second stages.

The Morris Water Maze Test

The Morris water maze experiment was carried out to assess spatial memory and memory function in rats (Vorhees and Williams, 2006). The MWM is a cylinder with a radius of 60 cm divided into four quadrants. The platform belongs to the fourth quadrant and is hidden 1 cm underwater. The training took place during the first 5 days. The sixth day was the experimental day. During the training period, the searching time for the platform

was 1 min, if the rat had not yet found the platform in 1 min, it was artificially dragged to the platform and stay for 30 s. During the experimental period, the platform was withdrawn, and the probe trial, the number of times and time of crossing the 5th quadrant were recorded.

Biochemical Analysis

Golgi Staining

After sacrificing the rats, the brains of 3 rats in each group were removed and placed immediately in 4% paraformaldehyde fixative (Servicebio, G1101) for more than 24 h and subsequently stained by Golgi staining (Golgi staining kit, Servicebio, G1069) and then photographed for analysis using the Image J 6.0 software [National Institute of Health (NIH), Bethesda, MD, United States] (Theer et al., 2014; Louth et al., 2017). Sholl analysis was used to analyze the dendrite length and branches. The CA1, CA2/3, and DG in HIP and PFC were analyzed. Briefly, the cell body of the neuron was used as the center in the 200× field of view. Then, concentric circles were made separated by a distance of 10 μm, and the sum of the intersection points of the dendrites and concentric circles (data of 10 concentric circles outside the cell) was counted. The total number of intersection points counted was considered to reflect the dendrites and the density of dendritic spines on the base dendrite in the field of 1000× was observed. Due to the uneven distribution of dendritic spines on the dendrites at all levels, observation was started from the first branch of the dendrite to the cell body, and the range of 30–90 μm was used for calculation. The number of internal dendritic spines is the density of dendritic spines per 10 μ m.

Western Blot Analysis

Samples of 30 mg of PFC and hippocampal tissues of 4 rats from each group were placed in 1.5 mL EP tubes. Then, for each 20 mg of tissue 200–250 μL of RIPA lysis buffer (Beyotime, Shanghai, China), containing 1% of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF), was added for tissue lysis. Subsequently, 2 high-pressure small steel balls were added to each EP tube, which were then placed a pre-cooled mill. The parameters used for grinding were 60 Hz and a grinding time of 60 s. After grinding to obtain a homogeneous mixture, the lysate was centrifuged at 12,000 rpm in a cold centrifuge at 4°C for 25 min. Afterward, the supernatant was carefully removed and aliquoted into 80 μL per tube. Protein concentration was determined by the bicinchoninic acid assay (BCA) method. Before loading the lysate samples onto the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel for protein separation, each lysate sample containing the same amount of protein was mixed with 5 × loading buffer and boiled for 10 min. Then, the proteins in each sample were separated on 8 and 10% SDS-PAGE gels and subsequently transferred onto a 0.22 or 0.45 μm polyvinylidene fluoride (PVDF) membrane. Then, after blocking the membrane with 5% skim milk for 2 h, the primary antibody against BDNF (CY5577, Abways Biotechnology Co., Ltd., Shanghai, China) or against H3K9me2 (ab1220, Abcam, Cambridge, United Kingdom) was added and the membrane was incubated overnight at 4°C (Berkel et al., 2019). Subsequently, after washing the membrane three times with tris-buffered saline Tween 20 (TBST) for 10 min each time, the secondary antibody was added, and incubated for 1 h at room temperature. The analysis of the protein bands grayscale values was performed by Image J software.

RNA Extraction and qRT-PCR

The brain tissues of 2 rats and a half of brain tissue of 1 rat from each group were used to test qRT-PCR. Total RNA was extracted from 20 mg of PFC and hippocampal tissue samples. Each tissue sample was placed in a 2 mL EP tubes containing 500 μL of Trizol (RR003, HONBIOTECH, Jinan, China) and two small steel balls. The tissue in each EP tube was ground using a pre-cooled grinder, at 60 Hz for 60 s, to obtain a homogeneous mixture. Then, 200 μL of chloroform were added to each homogenate mixture, followed by vigorous shaking, and incubation at room temperature for 5 min to fully extract RNA. Afterward, the tubes were centrifuged in a cold centrifuge and the supernatant was transferred into a new centrifuge tube, and mixed with an equal volume of isopropyl. At this step, the samples can be store at −80°C or the RNA extraction can be completed by centrifuging, discarding the supernatant, and washing the pellet with 75% ethanol (prepared in DEPC water) to obtain the RNA. The RNA concentration and integrity were determined using a spectrophotometer. The total RNA was reverse transcribed into cDNA using a reverse transcription kit (ReverTra Ace® qPCR RT Master Mix with gDNA Remover, FSQ-301, TOYOBO, Japan). The prepared cDNA was used to measure gene expression by qRT-PCR analysis using a Bio-Rad qRT-PCR system (Bio-Rad Laboratories, Hercules, CA, United States). The qRT-PCR generated data were used to determine the relative expression of the genes of interest using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The sequence of primers used is as follows: Rat-Bdnf-F 5′-GTCCCGGTATCAAAAGGCCA-3′; Rat-Bdnf-R 5′-ATCCTTATGAACCGCCAGCC-3′. Rat-β-actin-F 5′-CTCTGTGTGGATTGGTGGCT-3′, Rat-β-actin-R 5′-CGCAGCTCAGTAACAGTCCG-3′.

CHIP-qPCR

The PFC and hippocampal tissues of 2 rats and a half of brain tissue of 1 rat from each group were used to test CHIP-qPCR. Chromatin co-precipitation is a protein localization technique that analyzes binding to specific regions within the genome. Chromatin components can be selectively enriched using antibodies specific to the protein of interest. After enriching the protein of interest, pull-down technology is used to isolate the protein-interacting DNA regions. Then, qPCR is performed on the pull-down DNA to determine that the protein of interest is bound to the gene and its binding site is determined. According to CHIP kit (56383, Cell Signaling Technology, Inc., United states), this experiment was performed within 1 week sacrificing the rats. A total of 60–75 mg of tissue was manually cut into 1–2 mm pieces of tissue. Then, after adding CHIP cell lysis buffer, each tissue sample was manually ground into a single cell suspension. Afterward, ultrasonic lysis was performed after adding CHIP nuclear lysis buffer to obtain the ideal chromatin fragment of 150–1,000 bp. Then the immunoprecipitation reaction was performed, followed by de-crosslinking after overnight treatment with magnetic beads and antibodies. A DNA purification kit was used to obtain purified DNA to be used for CHIP-qPCR analysis and determine the

enrichment rate. This technology is used to further demonstrate the relationship between H3K9me2 and the *Bdnf* gene promoter, in order to determine whether H3K9me2 directly regulates the expression of the *Bdnf* gene and BDNF protein (Tsankova et al., 2004; Cowan et al., 2018). The experimental procedure of CHIP was showed in **Figure 2**. The sequence of primers used as follows: Bdnf-PF 5′-TGATCATCACTCACGACCACG-3′; Bdnf-PR 5′-CAGCCTCTCTGAGCCAGTTACG-3′.

Statistical Analysis

Quantitative data are expressed as the mean ± SEM (standard error of the mean). One-way analysis of variance (ANOVA)

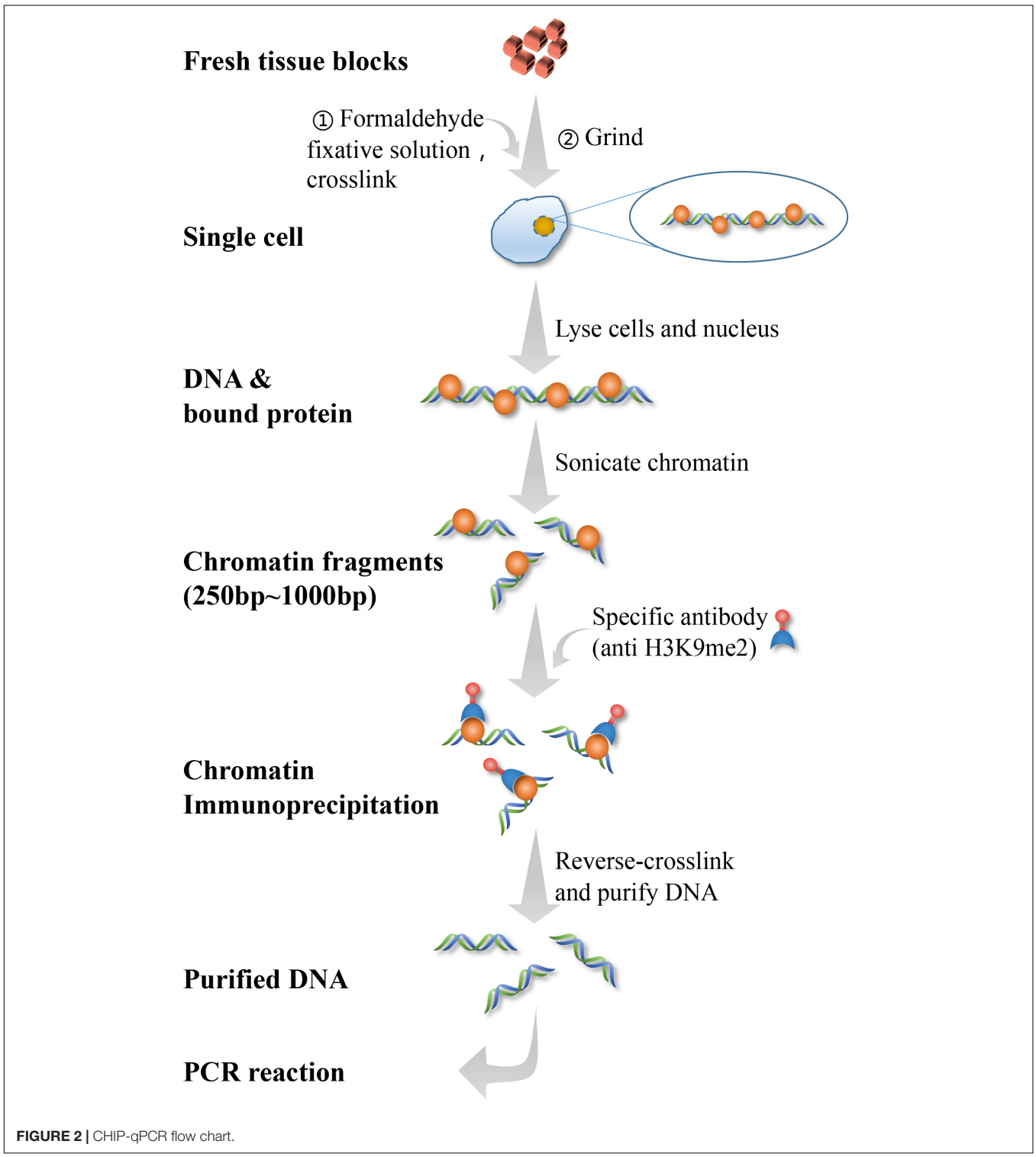

FIGURE 2 | CHIP-qPCR flow chart.

followed by Tukey's *post hoc* test was used for statistical analysis, except for the average escape latency of the MWM test. For the MWM test, the average escape latency in the 5 training days was used for three-way repeated-measures ANOVA. Differences were considered significant when the P value was < 0.05.

RESULTS

Behavioral Tests

Traumatic Stress Procedure and Effect of the Unc0642 on Exploratory and Anxiety-Like Behaviors Both in Adolescent and Adult Rats

The results of the OFT are shown in **Figure 3**. Traumatic stress reduced the number of crossings in the adolescent rats [$F(2, 33) = 11.20$, $P < 0.05$] (*post hoc*, $P = 0.002$) compared with the adolescent controls, and Unc0642 administration attenuated this behavioral change (*post hoc*, $P = 0.006$). However, there has no change in the number of crossings for adult rats [$F(2, 33) = 2.224$, $P > 0.05$] (*post hoc*, $P = 0.2726$) (**Figure 3A**). In addition, traumatic stress reduced the number of rearings in adolescent rats [$F(2, 33) = 9.103$, $P < 0.05$] (*post hoc*, $P = 0.0012$) and Unc0642 administration mitigated this behavior in adolescent rats (post hoc, $P = 0.0046$) compared with stressed rats. Meanwhile, traumatic stress decreased the number of rearings in the adult group [$F(2, 33) = 5.90$, $P < 0.05$] (*post hoc*, $P = 0.0037$) and Unc0642 treatment increased the number of rearings in this group (*post hoc*, $P = 0.0073$) (**Figure 3B**). Also, traumatic stress reduced the time spent in the center in adolescent rats [$F(2, 33) = 4.683$, $P < 0.05$] (*post hoc*, $P = 0.0382$)

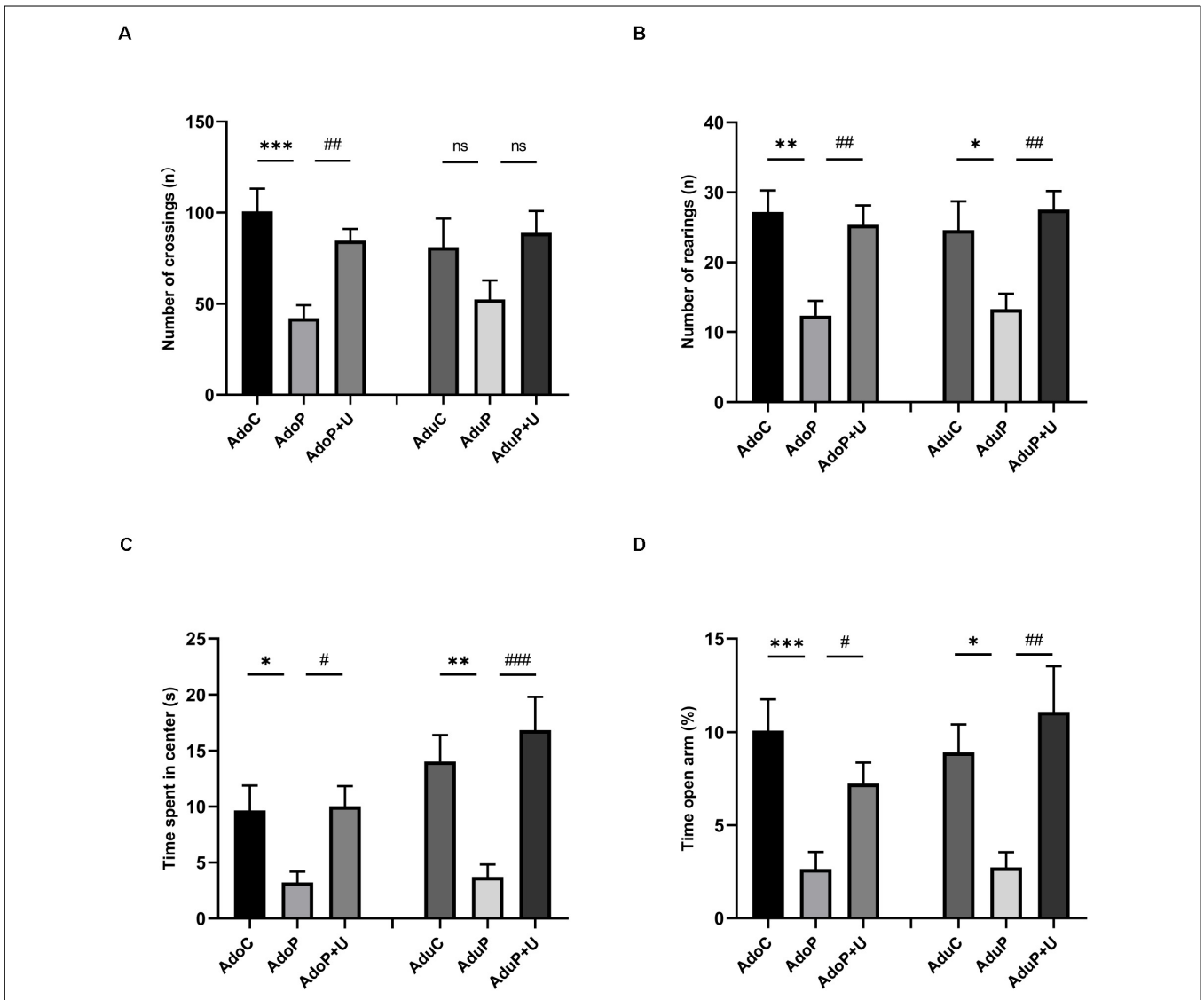

FIGURE 3 | Traumatic stress procedure and Unc0642 administration effect on depressive-like and anxiety-like behaviors both in adolescent and adult rats. **(A)** The number of crossings in OFT. **(B)** The number of rearings in OFT. **(C)** Time spent in center in OFT. **(D)** Time in open arm (%) in EPM test. Results were presented as mean ± SEM ($n = 12$ each group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; ns, none significant (Tukey's test).

and Unc0642 treatment alleviated this behavioral alteration in adolescent rats (*post hoc*, $P = 0.0271$). Additionally, traumatic stress decreased the time spent in the center in adult rats [$F(2, 33) = 9.27$, $P < 0.05$] (*post hoc*, $P = 0.0079$) compared with control rats and Unc0642 treatment attenuated this index (*post hoc*, $P = 0.0008$) (**Figure 3C**).

The results of the EPM test are shown in **Figure 3D**. Traumatic stress decreased the time in the open arm (%) in adolescent rats [$F(2, 33) = 8.59$, $P < 0.05$] (*post hoc*, $P = 0.0007$) and Unc0642 treatment with mitigated this behavior in adolescent rats (*post hoc*, $P = 0.0414$). Traumatic stress also decreased the time in the open arm (%) in adult rats [$F(2, 33) = 6.37$, $P < 0.05$] (*post hoc*, $P = 0.0407$) and Unc0642 treatment relieved this behavior in adult rats (*post hoc*, $P = 0.0044$) (**Figure 3D**).

Traumatic Stress Procedure and Effect of Unc0642 on Social Interaction Behaviors Both in Adolescent and Adult Rats

The results of the SIT are shown in **Figure 4**. Traumatic stress decreased the touching time in first stage in adolescent rats [$F(2, 33) = 5.75$, $P < 0.05$] (*post hoc*, $P = 0.0142$) and treatment with Unc0642 alleviated this behavior (*post hoc*, $P = 0.0182$) compared with adolescent stressed rats. Meanwhile, in adult groups, the effect of traumatic stress on the decrease

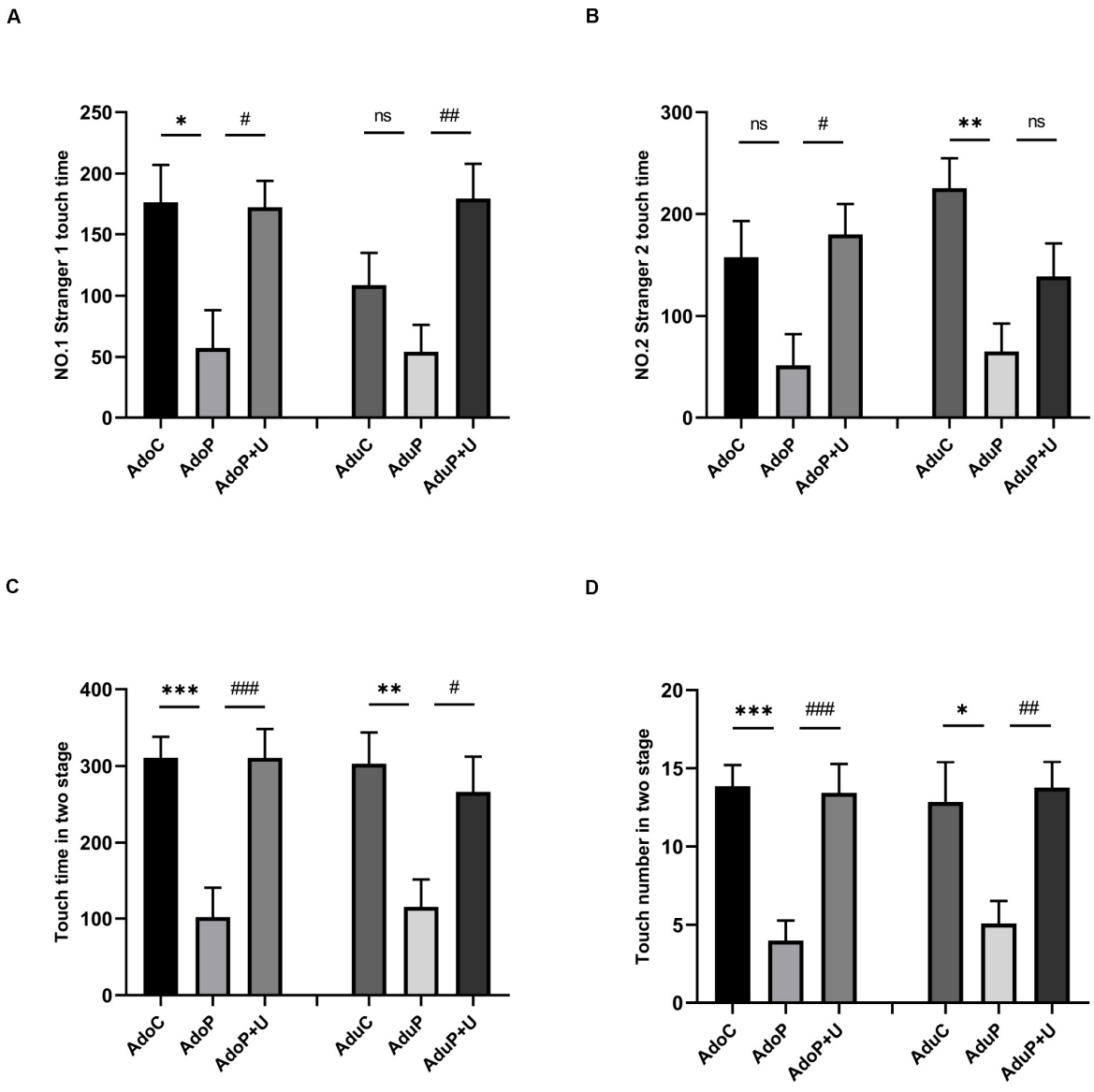

FIGURE 4 | Traumatic stress procedure and Unc0642 administration effect on social interaction changes both in adolescent and adult rats. **(A)** Stranger 1 touch time in first stage (NO.1) in three-chamber sociability test. **(B)** Stranger 2 touch time in second stage (NO.2) in three-chamber sociability test. **(C)** Touch time in two stages. **(D)** Touch number in two stages. Results were presented as mean ± SEM ($n = 12$ each group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; ns, none significant (Tukey's test).

of the touching time showed no significant statistical differences [$F(2, 33) = 5.877$, $P > 0.05$] (*post hoc*, $P = 0.3089$), but treatment with Unc0642 relieved the behavior in adult rats (*post hoc*, $P = 0.0047$) (**Figure 4A**).

In addition, traumatic stress decreased the touching time in second stage in adolescent rats, but the differences were not statistically significant [$F(2, 33) = 4.58$, $P > 0.05$] (*post hoc*, $P = 0.0640$), and Unc0642 administration mitigated this behavior in adolescent rats (*post hoc*, $P = 0.0209$) compared with stressed rats. Also, in adult groups, traumatic stress decreased the touching time in second stage in adult rats [$F(2, 33) = 7.151$, $P < 0.05$] (*post hoc*, $P = 0.0018$), but treatment with Unc0642 had no effect on relieving this behavior in adult rats (*post hoc*, $P = 0.2068$) (**Figure 4B**).

Traumatic stress also decreased the total touching time in first and second stages in adolescent rats [$F(2, 33) = 11.94$, $P < 0.05$] (*post hoc*, $P = 0.0005$) and Unc0642 administration attenuated this behavior in adolescent rats (*post hoc*, $P = 0.0005$) compared with stressed rats. In adult groups, traumatic stress decreased the total touching time in first and second stages [$F(2, 33) = 5.62$, $P < 0.05$] (*post hoc*, $P = 0.0088$) and treatment with Unc0642 alleviated this behavior in adult rats (*post hoc*, $P = 0.0404$) (**Figure 4C**).

Additionally, traumatic stress reduced the total touching number in first and second stages in adolescent rats [$F(2, 33) = 13.45$, $P < 0.05$] (*post hoc*, $P = 0.0002$) and treatment with Unc0642 attenuated this behavior in adolescent rats (*post hoc*, $P = 0.0003$) compared with stressed rats. In adult groups, traumatic stress reduced the total touching number in first and second stages [$F(2, 33) = 5.98$, $P < 0.05$] (*post hoc*, $P = 0.0217$) and Unc0642 administration relieved the behavior in adult rats (*post hoc*, $P = 0.0095$) (**Figure 4D**).

Traumatic Stress Procedure and Effect of Unc0642 on Spatial Learning and Memory Both in Adolescent and Adult Rats

The results of the MWM test are shown in the **Figure 5**. In adolescent groups, escape latency of the three groups in the training period was shortened day by day [$F(4, 132) = 78.22$, $P < 0.0001$], and there was no relation between time and stress [$F(8, 132) = 1.068$, $P > 0.05$]. Traumatic stress increased the time of the average escape latency on the first day ($P < 0.001$) and treatment with Unc0642 alleviated this behavior on the first day (*post hoc*, $P = 0.0350$) compared with stressed rats. Additionally, there were differences between these groups [$F(2, 33) = 7.50$, $P < 0.05$] (**Figure 5A**). In adult groups, the escape latency of the three groups in the training period was shortened day by day [$F(4, 132) = 37.47$, $P < 0.0001$], and there was no relation between time and stress [$F(8, 132) = 0.97$, $P > 0.05$]. In addition, traumatic stress increased the time of the average escape latency on the second day (*post hoc*, $P = 0.0020$) and Unc0642 administration not relieved this behavior on the second day (*post hoc*, $P = 0.1150$) compared with stressed rats, but Unc0642 administration relieved this behavior on the fifth day (*post hoc*, $P = 0.0460$). Additionally, there were differences between these groups [$F(2, 33) = 19.36$, $P < 0.0001$] (**Figure 5B**). Traumatic stress reduced the number of arrivals at the platform in adolescent rats [$F(2, 33) = 9.68$, $P < 0.05$] (*post hoc*, $P = 0.0020$) and treatment with Unc0642 mitigated this behavior in adolescent rats (*post hoc*, $P = 0.0013$) compared with stressed rats. Also, in adult groups, traumatic stress reduced the number of arrivals at the platform [$F(2, 33) = 7.66$, $P < 0.05$] (*post hoc*, $P = 0.0022$) compared with control rats and Unc0642 administration attenuated this behavior in adult rats (*post hoc*, $P = 0.0153$) (**Figure 5C**).

Biochemical Determination

Traumatic Stress Procedure and Effect of Unc0642 on Neuronal Morphological Changes of Golgi Staining Both in Adolescent and Adult Rats

The total number of intersections represent the length of each neuron and its degree of branch richness, which indicate the neuronal development. The longer length and the more branches reveal its better development. The results are shown in **Figure 6** (HIP-CA1) and **Supplementary Figure 1** (HIP-CA2/3 and DG), traumatic stress reduced the total number of intersections of the HIP-CA1 in adolescent rats [$F(2, 15) = 7.62$, $P < 0.05$] (*post hoc*, $P = 0.0260$) and treatment with Unc0642 relieved these changes in adolescent rats (*post hoc*, $P = 0.0057$) compared with stressed rats. In adult groups, traumatic stress reduced the number of intersections of the HIP-CA1 [$F(2, 15) = 4.21$, $P < 0.05$] (*post hoc*, $P = 0.0399$), but treatment with Unc0642 had no effect on relieving these changes in adult rats (*post hoc*, $P = 0.0959$) compared with stressed rats (**Figures 6A,C**). Although the same change tendency was observed in the CA2/3 and DG of the HIP, traumatic stress unchanged the number of intersections in the regions of CA2/3 (Ado: [$F(2, 15) = 5.18$, $P > 0.05$]; Adu: [$F(2, 15) = 0.57$, $P > 0.05$]) and DG (Ado: [$F(2, 15) = 3.36$, $P > 0.05$]; Adu: [$F(2, 15) = 0.96$, $P > 0.05$]) in both adolescent groups and adult groups (**Supplementary Figure 1**). In the PFC, traumatic stress also reduced the total number of intersections in adolescent rats [$F(2, 15) = 6.40$, $P < 0.05$] (*post hoc*, $P = 0.0378$) and Unc0642 administration mitigated these changes in adolescent rats (*post hoc*, $P = 0.0113$) compared with stressed rats. However, in the PFC of adult rats, traumatic stress reduced the total number of intersections [$F(2, 15) = 13.62$, $P < 0.05$] (*post hoc*, $P = 0.0005$) and the Unc0642 administration unimproved neuronal development (*post hoc*, $P = 0.6008$) compared with stressed rats (**Figures 6B,D**).

Traumatic Stress Procedure and Effect of Unc0642 on BDNF and BDNF mRNA Expression Both in Adolescent and Adult Rats

The results of the analysis of the effects of IFS and treatment with Unc0642 on BDNF and BDNF mRNA expression are shown in **Figure 7**. In the HIP, traumatic stress decreased the expression of BDNF protein in adolescent rats [$F(2, 12) = 24.03$, $P < 0.05$] (*post hoc*, $P < 0.0001$) and treatment with Unc0642 relieved the change in adolescent rats (*post hoc*, $P = 0.0005$) compared with stressed rats. In adult groups, traumatic stress decreased the expression of BDNF protein [$F(2, 12) = 8.39$, $P < 0.05$] (*post hoc*, $P = 0.0137$) and treatment with Unc0642 mitigated this change in adult rats (*post hoc*, $P = 0.0081$) (**Figure 7A**).

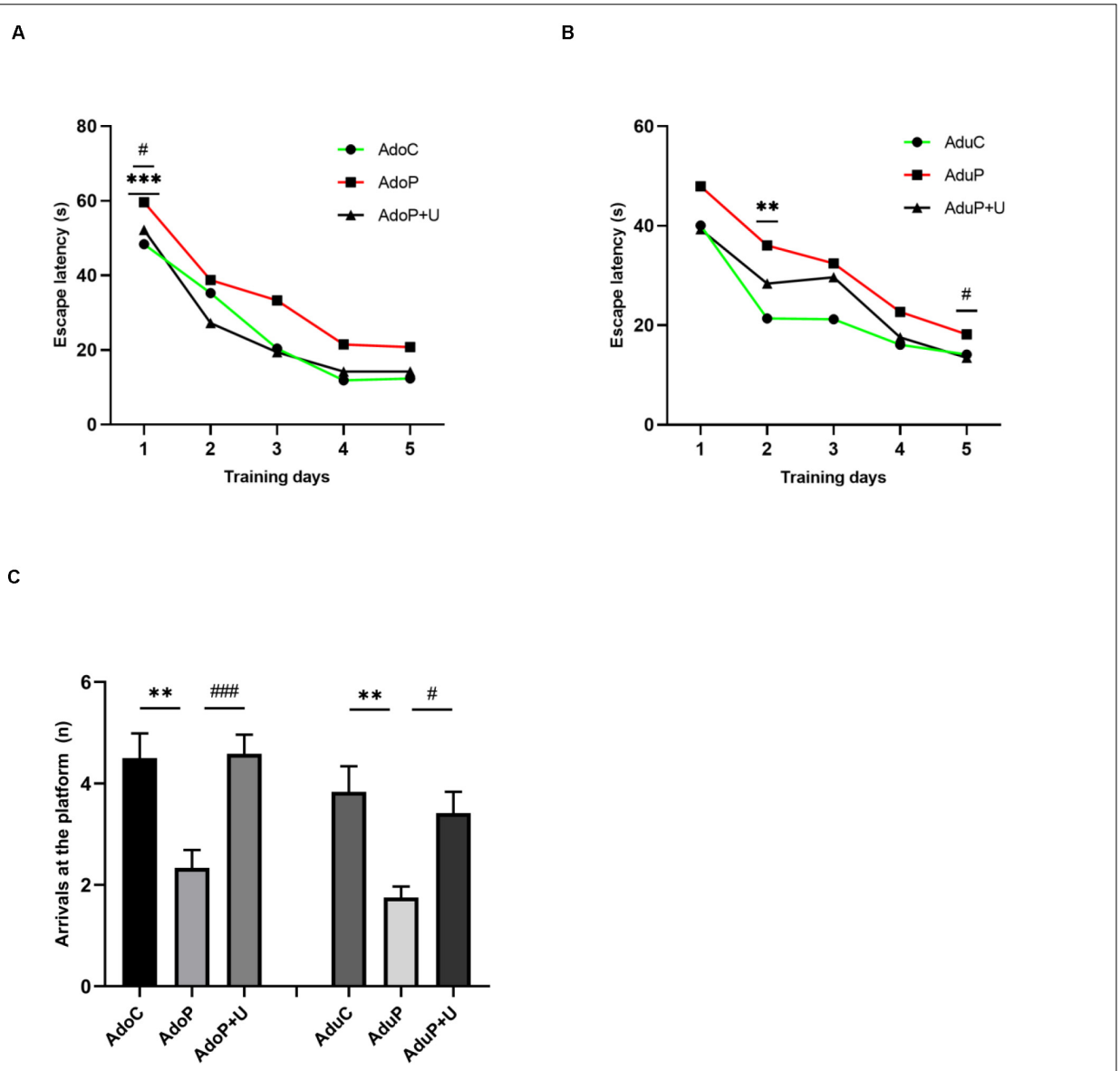

FIGURE 5 | Traumatic stress procedure and effect of Unc0642 on spatial learning and memory impairment both in adolescent and adult rats. **(A)** Escape latency in MWM test in adolescent rats. **(B)** Escape latency in MWM test in adult rats. **(C)** The number of arrivals at the platform in MWM test. Results were presented as mean ± SEM (n = 12 each group). $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ (Tukey's test).

In the PFC, traumatic stress decreased the expression of BDNF protein in adolescent rats [$F(2, 12) = 5.70$, $P < 0.05$] (*post hoc*, $P = 0.0268$) and Unc0642 administration attenuated this change in adolescent rats (*post hoc*, $P = 0.0038$) compared with stressed rats. In the PFC of rats in adult groups, traumatic stress decreased the expression of BDNF protein [$F(2, 12) = 7.08$, $P < 0.05$] (*post hoc*, $P = 0.0150$) and treatment with Unc0642 alleviated this change in adult rats (*post hoc*, $P = 0.0206$) compared with stressed rats (**Figure 7B**). Additionally, in the HIP, traumatic stress decreased the expression of BDNF mRNA in adolescent rats [$F(2, 6) = 12.74$, $P < 0.05$] (*post hoc*, $P = 0.0426$) and Unc0642 administration relieved this change in adolescent rats (*post hoc*, $P = 0.0060$) compared with stressed rats. In the HIP of rats in the adult groups, traumatic stress also decreased the expression of BDNF mRNA [$F(2, 6) = 13.65$, $P < 0.05$] (*post hoc*, $P = 0.0080$) and treatment with Unc0642 mitigated this change in adult rats (*post hoc*, $P = 0.0116$) compared with stressed rats (**Figure 7C**). In the PFC, traumatic stress also decreased the expression of BDNF mRNA in adolescent rats [$F(2, 6) = 14.33$, $P < 0.05$] (*post hoc*, $P = 0.0326$) and treatment with Unc0642 mitigated this change in adolescent rats (*post hoc*, $P = 0.0045$) compared with stressed rats. In the PFC of rats in adult groups, traumatic stress decreased the expression of BDNF mRNA [$F(2, 6) = 13.21$, $P < 0.05$] (*post hoc*, $P = 0.0174$) and Unc0642 administration alleviated this change in adult rats (*post hoc*, $P = 0.0071$) (**Figure 7D**).

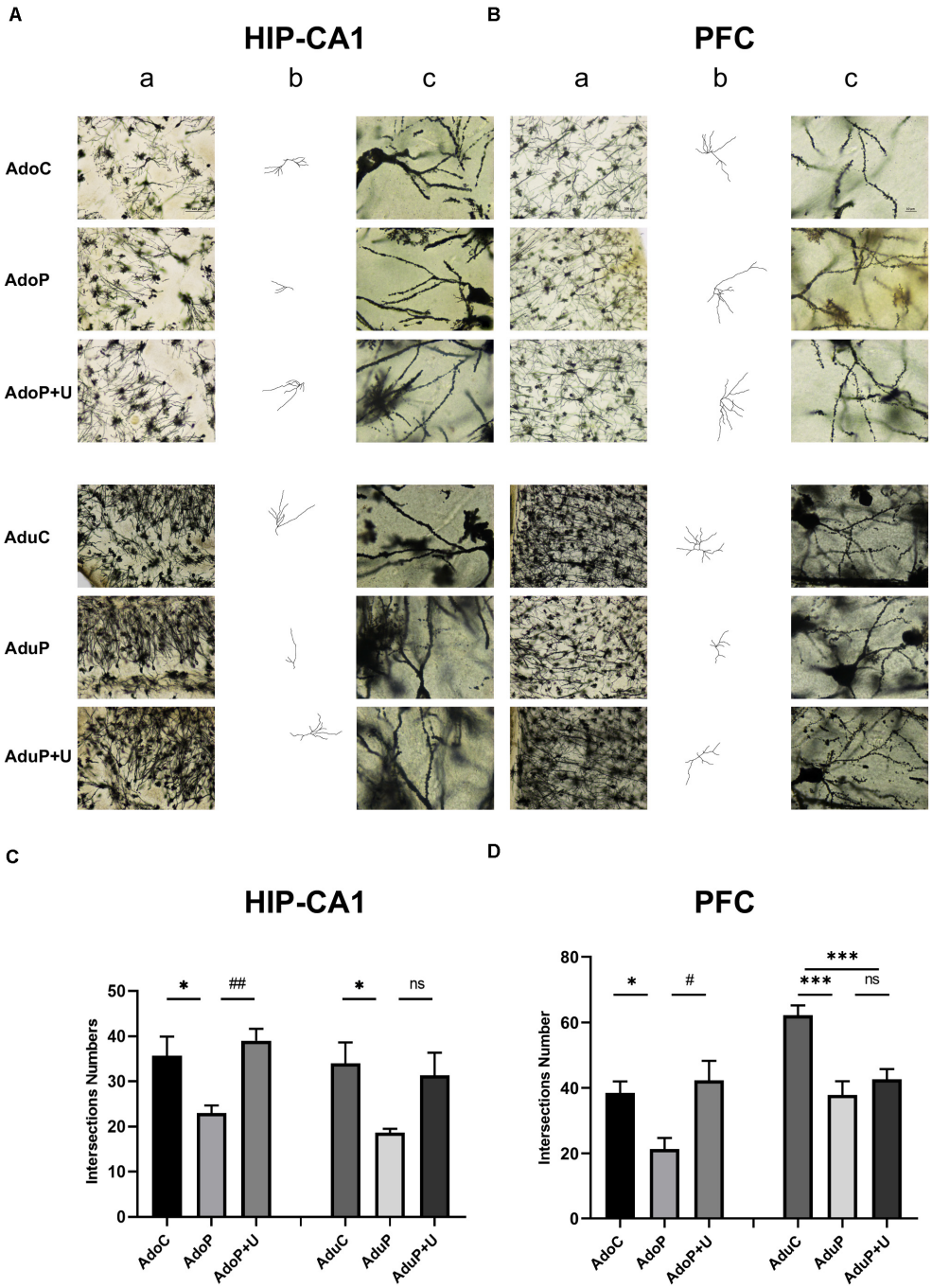

FIGURE 6 | Traumatic stress procedure and effect of Unc0642 on neuronal morphological changes of Golgi dyeing both in adolescent and adult rats. **(A)** Golgi dyeing in hippocampus-CA1 of adolescent and adult rats. **(B)** Golgi dyeing in prefrontal cortex of adolescent and adult rats. **(C)** Intersections number of adolescent and adult rats. **(D)** Intersections number of adolescent and adult rats. a. 200× in Golgi dyeing; b. Black and white picture; c. 1000× in Golgi dyeing. Results were presented as mean ± SEM (n = 3 each group). $^{*}p < 0.05$, $^{***}p < 0.001$ and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; ns, none significant (Tukey's test).

Traumatic Stress Procedure and Effect of Unc0642 on H3K9me2 Expression Both in Adolescent and Adult Rats

The results of the analysis of the effects of traumatic stress and treatment with Unc0642 on H3K9me2 expression are shown in **Figure 8**. In the HIP, traumatic stress increased the expression of H3K9me2 in adolescent rats [$F(2, 9) = 8.88$, $P < 0.05$] (*post hoc*, $P = 0.0208$) and treatment with Unc0642 mitigated this change (*post hoc*, $P = 0.0093$) compared with stressed rats. In the HIP of rats in adult groups, traumatic stress increased the

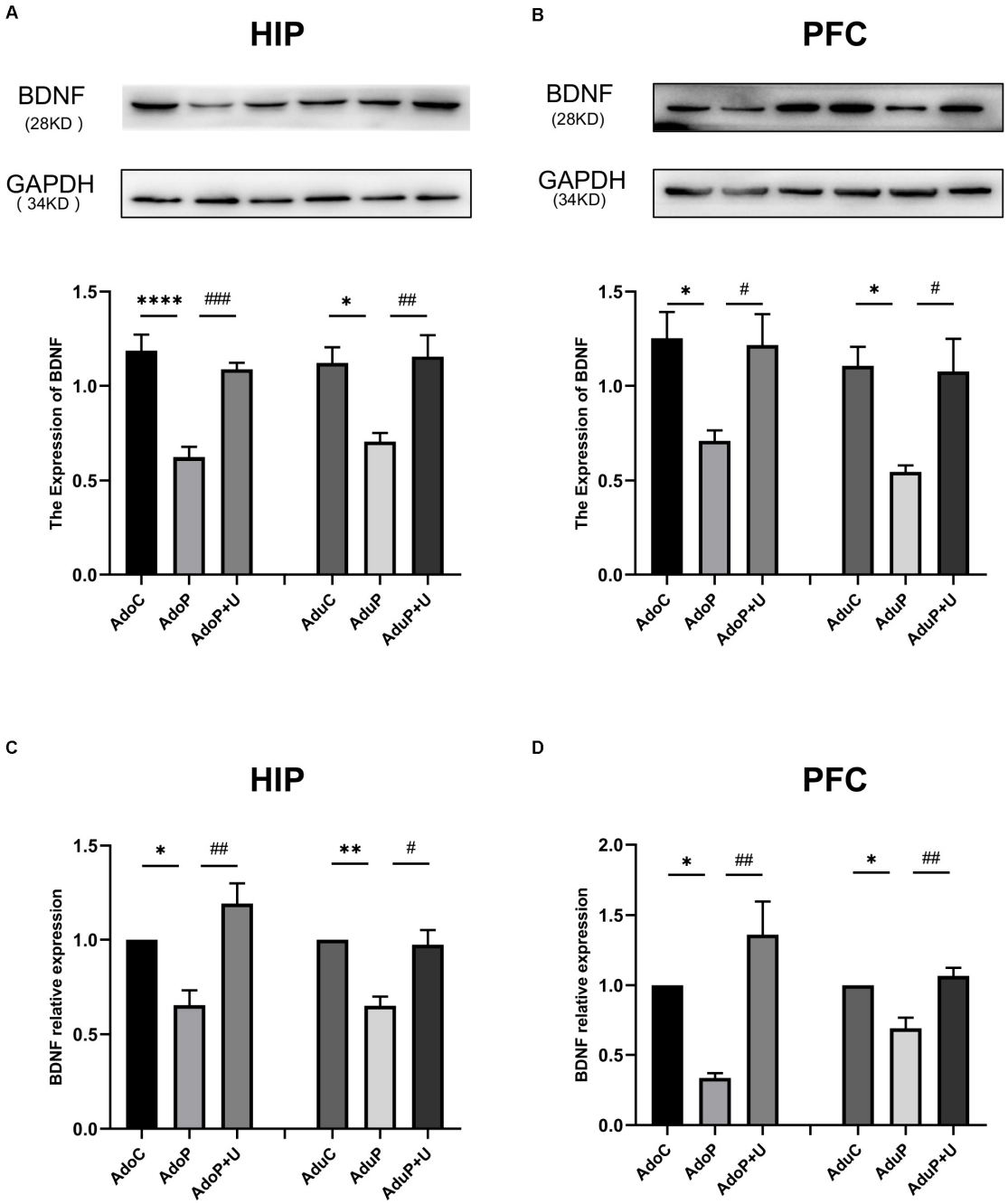

FIGURE 7 | Traumatic stress procedure and effect of Unc0642 on BDNF protein and BDNF mRNA expression both in adolescent and adult rats. Results were presented as mean ± SEM (n = 4/3 each group). Levels of BDNF protein in the hippocampus **(A)** and PFC **(B)** were quantified and normalized to GAPDH. Levels of BDNF mRNA in the hippocampus **(C)** and PFC **(D)** were quantified and normalized to β-actin. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (Tukey's test).

expression of H3K9me2 [$F(2, 9) = 9.516$, $P < 0.05$] (*post hoc*, $P = 0.0070$) and Unc0642 administration relieved this change in adult rats (*post hoc*, $P = 0.0199$) compared with stressed rats (**Figure 8A**). In the PFC, traumatic stress increased the expression of H3K9me2 in adolescent rats [$F(2, 9) = 5.87$, $P < 0.05$] (*post hoc*, $P = 0.0238$) and Unc0642 administration attenuated this change in adolescent rats (*post hoc*, $P = 0.0375$) compared with stressed rats. In the PFC of rats in adult groups, traumatic stress increased the expression of H3K9me2 [$F(2, 9) = 8.03$, $P < 0.05$] (*post hoc*, $P = 0.0155$) and treatment of Unc0642 mitigated this change in adult rats (*post hoc*, $P = 0.0197$) (**Figure 8B**).

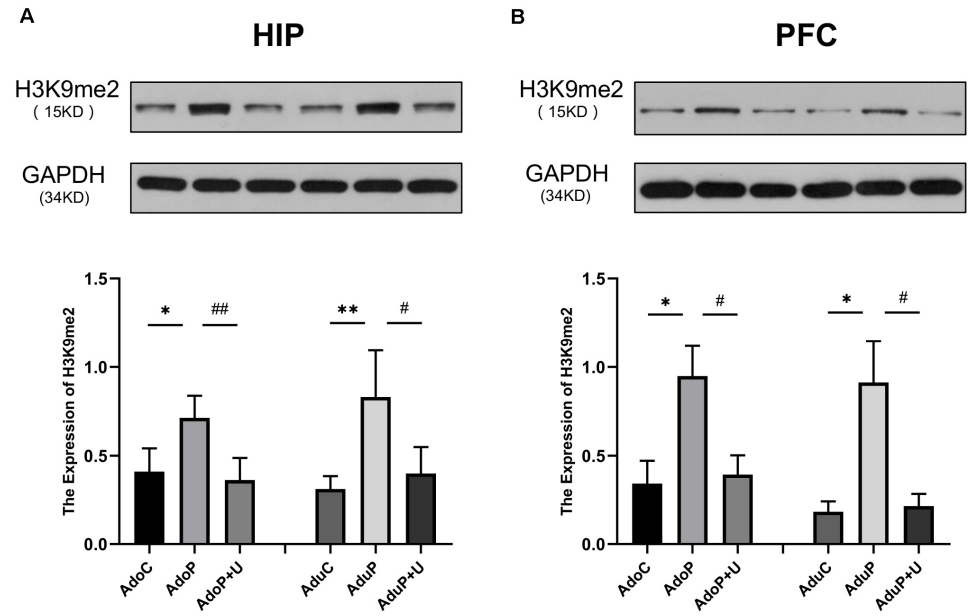

FIGURE 8 | Traumatic stress procedure and effect of Unc0642 on H3K9me2 expression both in adolescent and adult rats. Results were presented as mean ± SEM (n = 4 each group). Levels of H3K9me2 protein in the hippocampus **(A)** and PFC **(B)** were quantified and normalized to GAPDH. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (Tukey's test).

Traumatic Stress Procedure and Effect of Unc0642 on H3K9me2 Enrichment Both in Adolescent and Adult Rats

The epigenetic marker H3K9me2 can bind to the promoter region of the *Bdnf* gene and reduce the BDNF mRNA and BDNF protein expression levels. After stress, the expression of H3K9me2 is increased and leads to decreased expression of BDNF. In the HIP, traumatic stress increased the enrichment of H3K9me2 in adolescent rats [$F(2, 6) = 32.75$, $P < 0.05$] (*post hoc*, $P = 0.0013$) and treatment with Unc0642 decreased the enrichment of H3K9me2 in adolescent rats (*post hoc*, $P = 0.0009$) compared with stressed rats. In the HIP of rats in adult groups, traumatic stress increased the enrichment of H3K9me2 [$F(2, 6) = 15.27$, $P < 0.05$] (*post hoc*, $P = 0.0067$) and Unc0642 administration altered the enrichment in adult rats (*post hoc*, $P = 0.0079$) (**Figure 9A**). In the PFC, traumatic stress also increased the enrichment of H3K9me2 in adolescent rats [$F(2, 6) = 40.0$, $P < 0.05$] (*post hoc*, $P = 0.0004$) and treatment with Unc0642 decreased the enrichment of H3K9me2 in adolescent rats (*post hoc*, $P = 0.0009$) compared with stressed rats. Additionally, in the PFC of rats in adult groups, traumatic stress increased the enrichment of H3K9me2 [$F(2, 6) = 9.023$,

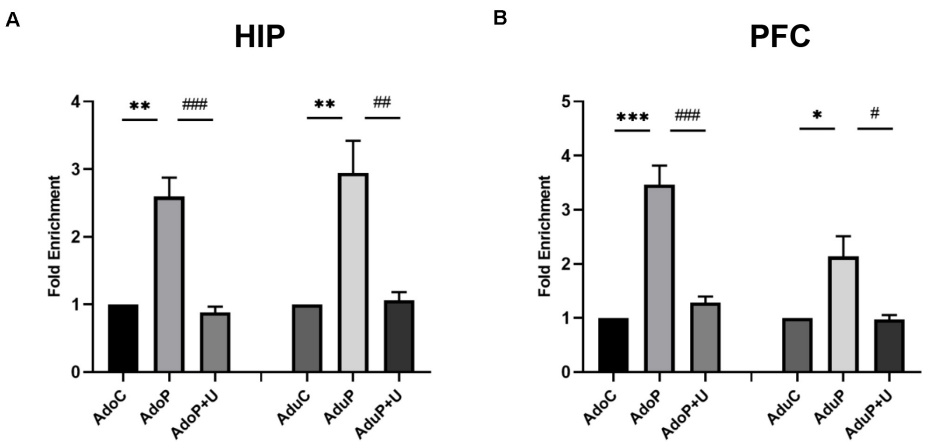

FIGURE 9 | Traumatic stress procedure and effect of Unc0642 on H3k9me2 enrichment both in adolescent and adult rats. **(A)**. Fold enrichment of H3K9me2 detected by CHIP; **(B)**. Fold enrichment of H3K9me2 detected by CHIP. Results were presented as mean ± SEM (n = 3 each group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (Tukey's test).

$P < 0.05$] (*post hoc*, $P = 0.0253$) and Unc0642 administration altered this change in adult rats (*post hoc*, $P = 0.0230$) in PFC (**Figure 9B**). Therefore, this result directly confirmed that H3K9me2 regulates the expression level of *Bdnf* gene.

DISCUSSION

The present study found that adolescent traumatic stress induced anxiety, exploratory behavior suppression, reduction of social exploratory behavior and impairment of spatial memory in rats. Such findings indicate that IFS induced post-traumatic stress-like behavioral disorders in young rats, and the symptoms could persist into adulthood in rats (Wang R. et al., 2018). However, most of the above behaviors were improved by treatment with Unc0642, a small molecule inhibitor of EHMT2, which could reduce the protein expression level of H3K9me2. Together, these findings suggest that the H3K9me2/BDNF axis is involved in the regulation of the pathogenesis of PTSD.

Golgi staining is a well-known staining method that can clearly reveal the neuronal morphology. Golgi staining can identify neurons from axons, dendrites and other nerves interlaced around it (Das et al., 2013). In this study, the classic Sholl analysis of Golgi stained neurons was used to analyze the total number of intersections, dendrite length, and density of individual neurons in the HIP and PFC (Theer et al., 2014). The results showed that there are significant differences in the total number of intersections among groups, which is reflected in the length of each neuron and its degree of branch richness. IFS reduced the total number of intersections in the CA1 of HIP and PFC in adolescent and adult rats compared with control rats, and treatment with Unc0642 mitigated these changes in adolescent rats. However, the neuronal morphological changes in the HIP and PFC of adult rats were not alleviated by treatment with Unc0642. The result in adult rats was consistent with those of previous study showing that antidepressants do not reverse stress-induced neurogenesis disruption (Lino de Oliveira et al., 2020). Also, these results suggested that early life stress induces non reversible morphological changes in certain subregion of the brain which affect the susceptibility of an individual to PTSD. It is noticed that traumatic stress unchanged the number of intersections in the CA2/3 and DG of HIP in both adolescent and adult rats which is consisted with the conclusion that the HIP subfields specific response to stressors (Chen et al., 2018).

Previous studies have shown that lower BDNF levels in the brain are closely related to anxiety-like behaviors (Yu et al., 2012). Furthermore, decreased BDNF and TrkB levels induced by acute and chronic stress could result in dysregulation of neural development and neural plasticity, which are involved in the pathogenesis of affective disorders, such as depression (Luo et al., 2019). In the present study, PTSD reduced protein and mRNA expression levels of BDNF both in the HIP and PFC, which is consistent with similar findings in previous studies. Together the results of behavioral tests and morphology analysis suggest that the reduction of BDNF expression in the HIP and PFC induced by early traumatic stress is involved in the etiology and susceptibility to PTSD (Burstein et al., 2018; Lee et al., 2019). However, the change occurring at the epigenetic level requires further validation by experimental studies.

Dimethylated histone H3 lysine 9 (H3K9me2) is a critical epigenetic mark for gene repression and silencing (Chen et al., 2006). H3K9me2 is altered by oxidative stress and metal exposure (Chervona and Costa, 2012). Further, studies suggest that H3K9me2/3 is a regulator of the gene expressing BDNF (Tsankova et al., 2004; Zhou et al., 2017). Present study analyzed the H3K9me2 protein expression level by western blotting and H3K9me2 enrichment using CHIP. The results demonstrated that IFS in adolescence increased H3K9me2 protein level in both adolescent and adult stressed rats, and the higher H3K9me2 protein level was relieved after Unc0642 administration. The results indicated that traumatic stress experiences alter the epigenetic regulation of BDNF expression in the HIP and PFC. In addition, the CHIP analysis results showed both adolescent and adult rats in the stressed groups had higher levels of H3K9me2 enrichment, while Unc0642 administration reduced such increases. The results confirmed that H3K9me2 regulates the level of expression of the *Bdnf* gene. This means that the epigenetic marker H3K9me2 can directly silence the transcription of the *Bdnf* gene, thereby decreasing the expression of its mRNA and protein, which confirmed the mechanism of epigenetic regulation in acute trauma stress model.

In summary, our study demonstrates that IFS in adolescent male rats can induce post-traumatic stress-like behaviors and such symptoms can persist into adulthood. In addition, stressed rats displayed fewer intersections, the indicator of the length of the dendrites and branches of neuron in the CA1 of HIP and PFC region. Moreover, IFS also decreased the expression level of BDNF mRNA and protein, and increased the levels of H3K9me2. However, the small molecule inhibitor Unc0642 improved both behavioral and molecular biological indicators. The results indicate that the H3K9me2/BDNF axis is involved in dendritic development and synaptogenesis in the brain, which is related to pathogenesis of PTSD.

LIMITATION

Present study has limitations. First, PTSD symptoms include depressive emotion (Wang W. et al., 2018; Richter-Levin et al., 2019), the sucrose consumption test and forced swimming test which can be used to evaluate the depression like behaviors of rats should be done. Second, previous studies show that the dorsal hippocampus has been implicated in memory function, while the ventral hippocampus has been implicated in stress and fear-related behaviors (Chen and Etkin, 2013; Chen et al., 2018). However, present study examined the expression of BDNF, BDNF mRNA and H3K9me2 enrichment in whole hippocampus, more precise study plan including injecting drug in the specific brain area should be done in the further. Finally, given our study only included male rats, the conclusion cannot be generalized to female population.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of Shandong University.

AUTHOR CONTRIBUTIONS

FP was involved in the study design and data interpretation. MZ performed the majority of the laboratory work and contributed to the analysis of the data and writing the manuscript. WW, ZZ, ZJ, and DL were responsible for the animal model and behavioral tests. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Differentially Expressed Genes in the Brain of Aging Mice With Cognitive Alteration and Depression- and Anxiety-Like Behaviors

Mengqi Li[1,2†], Songxue Su[2,3†], Weihua Cai[2,3], Jing Cao[2,3], Xuerong Miao[4], Weidong Zang[2,3], Shichao Gao[5], Ying Xu[5], Jianjun Yang[1,2], Yuan-Xiang Tao[6] and Yanqiu Ai[1,2*]*

[1] Department of Anesthesiology, Pain and Perioperative Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [2] Neuroscience Research Institute, Zhengzhou University Academy of Medical Sciences, Zhengzhou, China, [3] Department of Anatomy, College of Basic Medicine, Zhengzhou University, Zhengzhou, China, [4] Department of Anesthesiology and Intensive Care, Third Affiliated Hospital of Second Military Medical University, Shanghai, China, [5] Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, The State University of New York, Buffalo, NY, United States, [6] Department of Anesthesiology, New Jersey Medical School, Rutgers, The State University of New Jersey, Newark, NJ, United States

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United Kingdom

***Correspondence:**
Yuan-Xiang Tao
yuanxiang.tao@njms.rutgers.edu
Yanqiu Ai
aiyanqiu82@163.com

† These authors have contributed equally to this work

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Despite the great increase in human lifespan with improved medical care, the physiological and pathological changes such as memory and cognitive disorders and associated anxiety and depression are major concern with aging. Molecular mechanisms underlying these changes are little known. The present study examined the differentially expressed genes (DEGs) and the genes with differentially expressed isoforms in three brain regions, anterior cingulate cortex (ACC), amygdala and hippocampus, throughout the lifespan of mice. Compared to 2-month old mice, both 12- and 24-month old mice displayed memory and cognitive impairments in the Morris water maze, Y-maze, and novel object recognition tests and depression- and anxiety-like behaviors in the tail suspension, forced swimming, open field, and elevated plus maze tests. RNA sequencing analysis identified 634 and 1078 DEGs in ACC, 453 and 1015 DEGs in the amygdala and 884 and 1054 DEGs in hippocampus in the 12- and 24-month old mice, respectively. Similarly, many genes with differentially expressed isoforms were also identified in these three brain regions in the 12- and 24-month old mice. Further functional analysis revealed that many DEGs and the genes with differentially expressed isoforms in the ACC and amygdala were mapped to depression- and anxiety-related genes, respectively and that a lot of DEGs and the genes with differentially expressed isoforms in hippocampus were mapped to cognitive dysfunction-related genes from both 12- and 24-month old mice. All of these mapped DEGs and the genes with differentially expressed isoforms were closely related to neuroinflammation. Our findings indicate that these neuroinflammation-related DEGs and the genes with differentially expressed isoforms are likely new targets in the management of memory/cognitive impairment and emotional disorders during the aging.

Keywords: cognitive dysfunction, depression, anxiety, RNA sequencing, aging mice

INTRODUCTION

With rapid socio-economic development, the life expectancy of human being is increasing (Beard et al., 2016). However, age-related disorders such as Alzheimer's disease (AD) and dementia afflict vast majority of aged patients and become most significant public health issues and considerable challenges around the world (Kanasi et al., 2016). Indeed, the aging process may involve a series of behavioral and psychological dysfunction due to neurodegenerative changes in cell homeostasis and biological pathways (Gurkar and Niedernhofer, 2015). Clinical investigation indicates that aged patients with dementia often present clusters of behavioral symptoms such as memory and cognitive disorder, depression, anxiety, and neuropathic pain (Frisoni et al., 1999; Herrup, 2010; Salthouse, 2012; Harada et al., 2013). Although cognitive and functional impairment as the hallmark of aging is often emphasized, neuropsychiatric symptoms are also directly responsible for the reduced quality of life and the increased rates of disability in patients and their families. Current treatments for these age-related disorders are unsatisfactory and/or produce adverse effects at least in part due to incomplete understanding of molecular mechanisms underlying these disorders. Therefore, identifying the differentially expressed genes (DEGs) in the cognitive impairment-, depression-, or anxiety-associated brain regions of aging mice is a key step for searching new targets for novel treatments and preventative tactics for the age-related disorders.

Several brain regions such as hippocampus, amygdala and anterior cingulated cortex (ACC) are involved in the aging process and participate in the pathological processes of age-related cognitive and emotional disorders (Killgore and Yurgelun-Todd, 2004; Etkin et al., 2006; Liu W. et al., 2017; Martin et al., 2017; Ewbank et al., 2018; Piel et al., 2018). Indeed, the gene and protein networks among these three brain regions (Killgore and Yurgelun-Todd, 2004; Etkin et al., 2006; Ewbank et al., 2018; Piel et al., 2018) directly regulate cognitive dysfunction and depression/anxiety-like behaviors (Chao et al., 2016; Liu W. et al., 2017; Martin et al., 2017; Scalici et al., 2017; Yan et al., 2018). Although there are numerous hypotheses for understanding the pathogenesis of age-related memory deficits and emotional dysfunction, the dysregulation of genes and proteins in brain regions during aging has been widely acknowledged (Zhang et al., 2016; Alam et al., 2018; Holmes et al., 2018; Mehta et al., 2018). Several studies have identified DEGs between young- and aged-mice, but most of them focused on only one brain region and/or at a one-aged time point (Reichwald et al., 2009; Cribbs et al., 2012). Thus, it is imperative to obtain more thorough molecular profiles with aging by examining different gene changes in multiple brain sites throughout multiple time points during the lifespan.

To better understand the network of gene changes in the ACC, hippocampus and amygdala with aging, the present study focused on gene expression that changed in these three brain regions throughout the lifespan of mice. To this end, we employed the 2-, 12-, and 24-month old mice and first observed memory and cognitive performance, depression- and anxiety-like behaviors and the responses to nociceptive stimulation. We then collected ACC, amygdala and hippocampus from these mice and performed next-generation RNA sequencing with a higher sequencing depth and without mRNA poly-A tail selection. Finally, we analyzed and compared the transcriptome profiles in these regions among these different age mice. Our findings may provide novel information that may be used to identify new targets for the prevention and treatment of age-related neurodegenerative disorders.

MATERIALS AND METHODS

Animals

The 7-week old C57BL/6J wild-type male mice were obtained from Charles River Laboratory (Beijing, China) and Sino-British SIPPR/BK Lab (Shanghai, China) in this study. Animals were housed in a temperature-controlled room and exposed to a standard 12 h light-dark cycle and normal illumination environment. Three groups of different age mice (2-, 12-, and 24-month old; 8 mice/group) were used. The animals had at least 1 week for acclimation to facility environment before behavioral tests, which were carried out in a quiet room between 10:00 a.m. and 4:00 p.m. (daytime). The mice were acclimated to experimental apparatus for 45 min before the tests. All behavioral tests were done within 1 week. All procedures were conducted in accordance with the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain and approved by the Animal Care and Use Committee of Zhengzhou University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Open Field Test

The open field test (OFT) was performed to evaluate locomotion activity and anxiety-like behavior as described previously (Sun et al., 2016). The open field apparatus consisted of a gray Plexiglas box (50-cm long × 50-cm wide × 40-cm high), which was divided into 16 virtual squares (12 peripheral squares and 4 central squares). The light source was 1.8 m above the ground. The light intensity was 50 W. Briefly, mice were allowed to explore the arena for 5 min freely. Time spent in the central squares, distance in the central squares, numbers of entries, and the total distance traveled were recorded and analyzed by the video tracking system of SMART 3.0 (Panlab Harvard Apparatus, Spain). After each test, the open-field arena was cleaned with 10% bleach and 75% ethanol (Davalli et al., 2016; Fougere et al., 2017; Hekmatimoghaddam et al., 2017; Liu E. et al., 2017; Costa-Ferreira et al., 2019; Zhou et al., 2019).

Elevated Plus-Maze Test

The elevated plus-maze (EPM) test was used for assessing anxiety-like behavior (Rice et al., 2019). An elevated plus-shaped maze (50 cm above the floor) consisted of a central platform (5-cm long × 5-cm wide), two open arms (30-cm long × 5-cm wide), and two closed arms (30-cm long × 5-cm wide × 15-cm high). The open and close arms were situated opposite to each other. In the testing session, each mouse was placed in the central platform facing one of the open arms and allowed to explore freely for

5 min in a dim room. The percentage of open arm entries was calculated as the number of open-arms entries divided by total open and closed arms entries. The percentage of time spent in open arms was calculated as time spent in the open arms divided by total time (Franco et al., 2017; Klinger et al., 2019).

Forced Swimming and Tail Suspension Tests

Forced swimming and tail suspension tests (FST and TST) were used to measure depression-like behaviors by recording the immobility time of animals. In the FST (Inestrosa and Varela-Nallar, 2014), the mouse was placed individually into the transparent beakers (20-cm diameter by 25-cm height) for 6 min with a water depth of 15 cm at 24–25°C. The duration of cumulative immobility was recorded during the last 4 min within the 6-min testing period. The immobility was identified as the mouse floated in the water without struggling. For the tail suspension testing (Wadhwa et al., 2018), mice were suspended through the taped and affixed approximately 1 cm from the tip of the tail, 50 cm above the floor. All mice were suspended for 6 min in each session, and the immobility period was recorded in the last 4 min of the testing period. Mice were considered to be immobile only when they were passive and completely stationary. The experimental procedures were recorded by digital video-camera.

Novel-Object Recognition Test

The novel-object recognition (NOR) test was conducted in a square gray Plexiglas test box (50-cm long × 50-cm wide × 40-cm high) to test learning and memory (Lueptow, 2017). Animals were habituated to the arena for 5 min every day for 2 days before the test. Each mouse was allowed to explore two identical objects (A1 + A2) placed in the arena for 5 min. Three trials were conducted with an inter-trial interval of 15 min. Animals were kept in cages until the next trial at the intervals. Recognition index from the third trial during training session was used to rule out mouse preference for two objects. Mice were put back to the center of the arena and explore for another 5 min with a novel object (B; replacing A2) at 3 and 24 h after the third trial. Watching, licking or touching the object with forepaws and facing the object at the distance of approximately 2 cm were considered as object exploration. Behaviors were recorded and analyzed by the video tracking system of Smart 3.0 (Panlab Harvard Apparatus, Spain). Recognition index was calculated as the investigation time novel object / (investigation time novel object + investigation time familiar object) (Lueptow, 2017; Costa-Ferreira et al., 2019; Zhou et al., 2019).

Y-Maze Spontaneous Alternation Test

Spatial working memory was tested in the Y-maze spontaneous alternation task (Liu E. et al., 2017). Spontaneous alternation performance was assessed by a gray Plexiglas 3-arm Y-maze (each arm: 30-cm long × 7-cm wide × 15-cm high) under weak light conditions. Mice were randomly placed in one of the three arms and allowed to explore freely for 5 min. The number and the sequence of the visited arms were recorded. The number of arm entries and the percentage of spontaneous alternation [number of alternation / (number of total arm entries-2) × 100%] was calculated.

Morris Water Maze Test

Morris water maze (MWM) was another approach to evaluate spatial learning in rodents, as described previously (Vorhees and Williams, 2006; Xu et al., 2018). Mice were trained in a water maze (120 cm in diameter, 50 cm in depth) filled with water (25 cm in depth,23 ± 0.5°C). The pool was divided into four virtual quadrants, each with a cue to assist the mice to find the hidden platform (8.5 cm in diameter, 24 cm in height) that was submerged 1 cm under the water in the middle of one of the four quadrants. Each mouse was placed in a quadrant other than where the hidden platform was located, facing the wall of the pool, and was given 60 s to find the platform and 15 s to stay on it. Animals that did not find the platform were gently guided and placed on it during the learning test. Mice were trained for five consecutive days (three trials per day with an inter-trial interval of 20 min), and mean latency (time taken for the mouse to find the platform) from three trails per day was calculated. At 1 or 24 h after the training, the hidden platform was removed and learning was assessed through measuring the latency taken for the mouse to find the place where the hidden platform was located before and the number of crossing this place within 60 s (Klinger et al., 2019; Zhou et al., 2019).

Mechanical Test

Paw withdrawal frequencies in response to mechanical stimuli were tested as described before (Xu et al., 2018). Briefly, each mouse was placed individually in a Plexiglas chamber on an elevated mesh. Two calibrated von Frey filaments (0.07 g and 0.4 g; Stoelting, Kiel, WI, United States) were applied to the hind paw for approximately 1 s, respectively. Each stimulation was repeated ten times to both hind paws. The occurrence of paw withdrawal in each of these ten trials was expressed as a percent response frequency [(number of paw withdrawals/10 trials) × 100 = % response frequency].

Thermal Test

Paw withdrawal latencies to noxious thermal stimulation were examined as previously described (Porsolt et al., 1978). Each mouse was placed in a Plexiglas chamber on a glass plate. Radiant heat from the Plantar Test Instrument (Ugo Basile 37370, Italy) was applied by aiming a beam of light through the glass plate to the middle of the plantar surface of each hind paw. When the mouse lifted its foot, the light beam was cut off. The length of time between the start of the light beam and the foot lift was defined as the paw withdrawal latency. Each trial was repeated five times at 5-min intervals for each side. A cut-off time of 20 s was used to avoid tissue damage to the hind paw.

RNA Extraction

On the final day, the mice were sacrificed immediately after behavioral tests. The anterior cingulate cortex (ACC), hippocampus and amygdala were collected in the tubes

containing RNAlater (Ambion, Austin, TX, United States). Total RNA was extracted using the miRNeasy kit with on-column digestion of genomic DNA (QIAGEN, Valencia, CA, United States). RNA concentration was measured using the NanoDrop 2000 Spectrophotometer (Thermo Figher Scientific, Wilmington, DE, United States). The ratios of A260/280 nm were between 1.97 and 2.08 in all samples. RNA integrity was assessed using RNA Nanochips in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States). RNA integrity number (RIN) was between 7.5 and 8.4.

RNA Sequencing

Three biological replicates (three mice) from each age group were used in the RNA-seq analysis. Total RNA extracted above was subjected to rRNA depletion by Ribo-Zero rRNA Removal (Human/Mouse/Rat) Kit (Illumina, San Diego, CA, United States). Strand-specific RNA libraries were prepared using TruSeq Stranded Total RNA Sample Preparation Kit (Illumina) without poly-A selection. All assays were performed according to the manufacturer's instructions. Sequencing was carried out at the Illumina Nova6000 plate High Output Model (Illumina, San Diego, CA, United States) (Hrdlickova et al., 2017), in a 2 × 150 bp paired-end configuration, with a total of more than 2,666 M reads per lane (at least 40 M reads per sample). All sequencing data are available in NCBI database (accession number: SRP271007).

Bioinformatics Analysis

Nine samples from ACC, hippocampus, and amygdala (three repeats/region) were subjected to multiplexing, sequencing, differential gene expression, and transcript expression analysis. Briefly, the sequences were first trimmed in quality using trimmomatic-0.32 (Minimal length 50 bp, leading and trailing Phred Q 30). The resulting sequencing data were then mapped to the musculus genome sequence version GRCm38.72, downloaded from ENSEMBLE. Gene hit counts and reads per kilobase per million mapped reads (RPKM) were calculated for each gene to determine expression levels. DEGs and the genes with differentially expressed isoforms were filtered to *P*-value 0.05 and log base twofold change (LFC) ≥ 1 or ≤ -1. The above RNA-Seq analyses, including mapping, read counts and expression analysis, were carried out within the CLCbio software environment (CLC Genomics Workbench 7.0.2, CLC genomics Server). Mapped reads were visualized on the UCSC browser using bigwig files converted from bam files. The heatmap was generated using Heatmaper[1]. To analyze the functions of DEGs and the genes with differentially expressed isoforms, we used GeneCards database[2] and CTD database[3] to map them with depression and anxiety in the ACC and amygdala as well as with cognitive dysfunction in hippocampus. They were also mapped to inflammation, apoptosis, oxidative stress, synaptic plasticity, glutamate-receptor pathway, and DNA methylation.

[1]http://www.heatmapper.ca/expression/

[2]https://www.genecards.org/

[3]http://ctdbase.org/

Quantitative Real-Time RT-PCR

Total RNA was reverse-transcribed using the ThermoScript reverse transcriptase (Invitrogen) according to the manufacturer's instructions with the oligo (dT) primers. cDNA was amplified by real-time PCR using the primers listed in **Supplementary Table 1** (Integrated DNA Technologies, Coralville, IA, United States). Each sample was run in triplicate in a 20 μl reaction with 250 nM forward and reverse primers and 10 μl of Advanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, United States). Reactions were performed in a BIO-RAD CFX96 real-time PCR system. The cycle parameters were set as follows: an initial 3 min incubation at 95°C, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Ratios of mRNA levels from 12- or 24-month old mice to mRNA levels from 2-month old mice were calculated using the ΔCt method (2-ΔΔCt) at a threshold of 0.02. All data were normalized to an internal housekeeping gene *Gapdh* (Wen et al., 2020).

Functional Enrichment Analysis of Differentially Expressed Genes

For biological pathway analysis, approximately 634, 453, and 884 DEGs respectively from ACC, amygdala, and hippocampus of the 12-month old mice and 1078, 1015, and 1054 DEGs, respectively from ACC, amygdala, and hippocampus of the 24-month old mice were selected based on significantly differential changes in their expression as compared with the 2-month old mice. Panther Classification System database system was used to categorize the differential expression genes. (© 2015, Paul Thomas[4]). To comprehensively analyze the functions of the DEGs in the ACC, amygdala and hippocampus, all DEGs (including the upregulated genes and downregulated genes) were analyzed using the gene ontology enrichment analysis from the Database for Annotation, Visualization, and Integrated Discovery (DAVID[5]).

Protein-Protein Interaction Network Construction

STRING (version: 11.0) database[6] was used to predict the interaction between the proteins encoded by the DEGs. The protein–protein interaction (PPI) network was built using Cytoscape software (version: 3.6.0[7]) (Liu et al., 2019). The connection degree of each node was calculated through the centiscape plugin, and the first 50 genes that were selected based on connection degree were used to construct networks. Nodes were excluded from the network due to interaction with other nodes. The size of the node was determined by the degree of connection degree in the network, and use red, blue, and purple colors to mark the genes related to inflammation, apoptosis, and both inflammation and apoptosis, respectively.

[4]pantherdb.org

[5]http://david.abcc.ncifcrf.gov/

[6]https://string-db.org/cgi/

[7]www.cytoscape.org/

Correlational Analysis Between mRNA Levels of DEGs and Mouse Behaviors

Three DEGs were selected from ACC, amygdala and hippocampus, respectively, of 2- and 12-month old mice according to their scores between the genes and depression, anxiety and cognitive dysfunction based on the GeneCards database[2] and CTD database[3]. A correlation analysis between the expressional levels of these DEGs from 2- and 12-month old mice and behavioral scores from these corresponding mice was performed via the Pearson's method using the SPSS Software (IBM SPSS Statistics 21.0).

Statistical Analysis

All data were collected randomly and expressed as mean ± SEM. The data were statistically analyzed with two-tailed, unpaired Student's *t*-test or one- or two-way ANOVA with repeated measures. When ANOVA showed a significant difference, the pairwise comparison between means was tested by the *post hoc* Tukey method. *P*-values less than 0.05 were considered statistically significant.

RESULTS

General Appearance and Behaviors in Aging Mice

As shown in **Supplementary Figure 1**, the body weights of 12- and 24-month old mice were increased by 1.24-fold and 1.32-fold, respectively, as compared with 2-month old mice. Additionally, there were differences in the general appearance among these three groups of mice. The 12- and 24-month old mice exhibited some aging appearances, including slight body bending with hair loss and lacking luster. The 24-month old mice displayed lightly unstable gait and reduced spontaneous activity. There were no visible differences in the gait and spontaneous activity between the 12- and 2-month old mice.

Impaired Cognitive and Memory Abilities in Aging Mice

Cognitive and memory abilities in three groups of mice were observed by novel object recognition (NOR), Y-maze spontaneous alternation, and MWM tests. In the NOR test, the mice from 2-, 12-, and 24-month old did not exhibit any differences in basal precognitive behavior in the training session (**Figure 1A**). However, 3 and 24 h later after training, 12- and 24-month old mice displayed a significantly reduced recognition in exploring new objects as compared with the 2-month old mice. Consistently, the Y-maze test showed that the percentages of spontaneous alternation were robustly decreased in 12- and 24-month old mice compared with those of 2-month old mice (**Figure 1B**), indicating the decline in spatial memory. In addition, a significant reduction in the total number of arm entries was found in 24-month old mice, but not in 12-month old mice (**Figure 1C**). This indicates that 24-month old mice may have abnormal locomotor activity. In the MWM test, 12-month old mice displayed a longer latency to reach the platform compared to 2-month old mice in the 5th trial in the learning curve (**Figure 1D**). Spatial and working memory was further determined by removing the platform. As shown in **Figures 1E–H**, 12-month old mice exhibited the significantly lower numbers of crossing the place where the escape platform was located previously and the prolonged escape latencies at 1 and 24 h after training, suggesting the worse memory retention in 12-month old mice. As expected, we found that the total distances traveled at 1 and 24 h probe trials did not differ between 2- and 12-month old mice (**Figures 1I,J**), indicating that the performance differences observed above did not result from the changes in overall activity. The 24-month old mice were not employed in the MWM test considering the high risk of this test due to the reduced spontaneous activity and impaired locomotor function (see below) in these mice.

Depression- and Anxiety-Like Behaviors in Aging Mice

Forced swimming tests and tail suspension tests are the classical methods to screen the rapid behavioral changes in stressful situations. Immobility time was used as an indicator of depression in these tests. As shown in **Figures 2A,B**, immobility times in both 12- and 24-month old mice were significantly prolonged as compared with 2-month old mice, demonstrating that the aging mice were more likely to give up struggling in both tests due to despair.

To determine the anxiety-like behavior in aging mice, we performed the open field and EPM tests. In the OFT, we found that the overall distance traveled was conspicuously decreased in 24-month old mice, but not in 12-month old mice (**Figure 2C**), suggesting the impaired locomotor activity in 24-month old mice. Further observations showed the reduced exploration activity in 12- and 24-month old mice evidenced by the considerable reductions in distance traveled and time spent in the center area (**Figures 2D,E**), as well as in numbers of entering the central area (**Figure 2F**). Similar to whatever we observed in OFT, the EPM test showed that both the percentage of time spent in the open arms (**Figure 2G**) and the percentage of open arm entries were much lower in 12- and 24-month old mice than those in 2-month old mice (**Figures 2G,H**). As expected, the significantly decreased number of total entries to both open and closed arms in 24-month old mice, but not 12-month old mice, was seen (**Figure 2I**), further demonstrating impaired motor function only in 24-month old mice. Taken together, these findings indicate the depression- and anxiety-like behaviors in aging mice.

Basal Nociceptive Behavioral Responses in Aging Mice

To understand comprehensive phenotypes with aging, we examined basal nociceptive behavioral responses to mechanical and thermal stimuli in these three different ages of mice. As

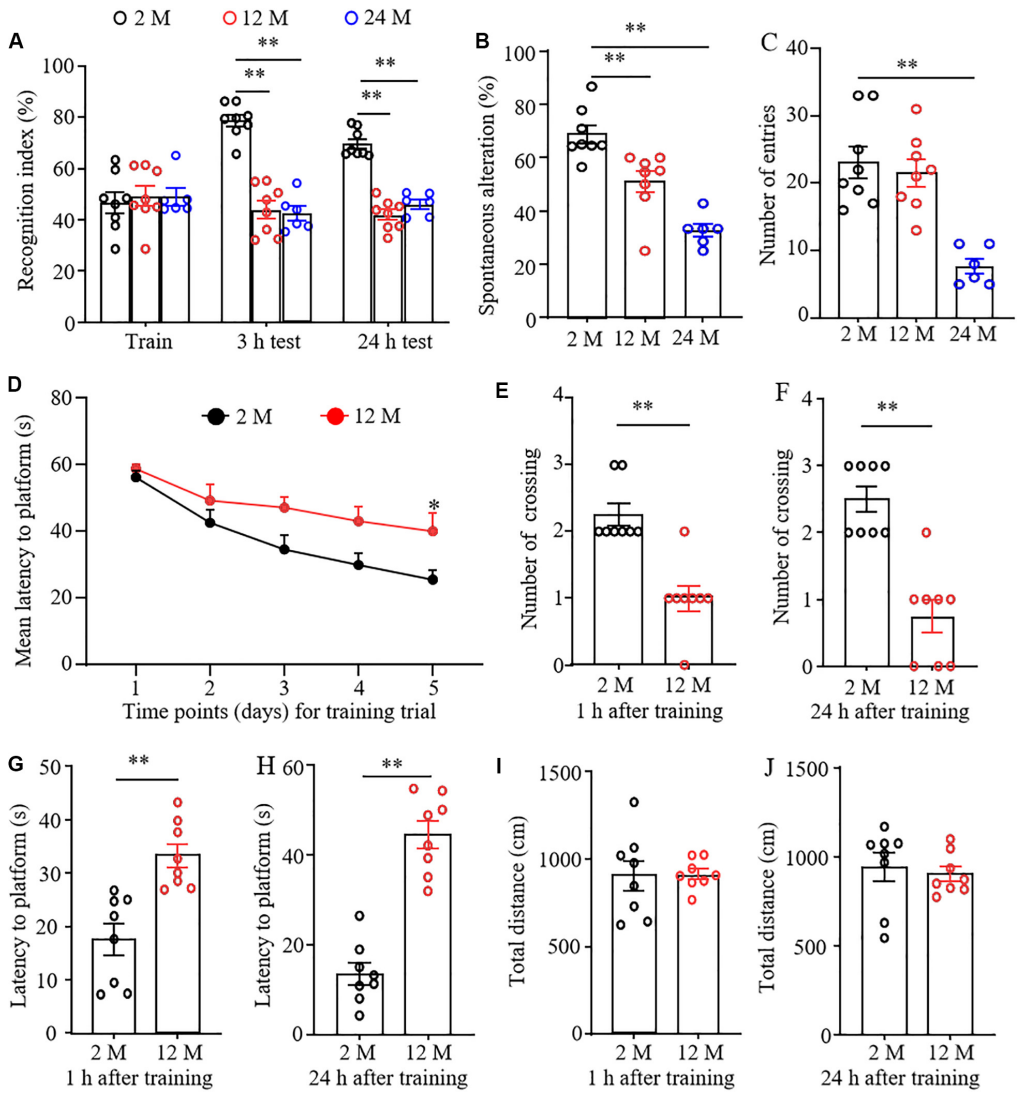

FIGURE 1 | The cognitive dysfunction in aging mice. $n = 8$ for 2- or 12-month mice, $n = 6$ for 24-month mice. Data were shown as mean ± SEM. **(A)** The recognition indexes were significantly reduced in 12- and 24-month old mice as compared to 2-month old mice at 3 and 24 h after the training session in the novel object recognition test. $^{**}P < 0.01$ versus the corresponding 2-month old mice by two-way ANOVA with repeated measures followed by *post hoc* Tukey test. **(B)** The percentages of spontaneous alterations were significantly reduced in 12- and 24-month old mice as compared to 2-month old mice in the Y maze test. $^{**}P < 0.01$ versus the corresponding 2-month old mice by one-way ANOVA with repeated measures followed by *post hoc* Tukey test. **(C)** The number of arm entries was significantly reduced in 24-month old mice, but not in 12-month old mice in the Y maze test. $^{**}P < 0.01$ versus the corresponding 2-month old mice by one-way ANOVA with repeated measures followed by *post hoc* Tukey test. **(D)** The learning curve was examined for 5 days in the Morris water maze test. $^{*}P < 0.05$ versus 2-month old mice at the corresponding time point by two-way ANOVA with repeated measures followed by *post hoc* Tukey test. **(E,F)** Platform crossing times in the probe trial of the MWM test at 1 **(E)** and 24 h **(F)** after the training session. $^{*}P < 0.01$ versus 2-month old mice by two-tailed unpaired Student's *t*-test. **(G,H)** The average escape latency to reach the previous platform location was examined at 1 **(G)** and 24 h **(H)** after the training session. $^{**}P < 0.01$ versus 2-month old mice by two-tailed unpaired Student's *t*-test. **(I,J)** Total distance of movement was examined at 1 **(I)** and 24 h **(J)** after the training session. Two-tailed unpaired Student's *t*-test.

shown in **Figure 3A–D**, there were no significant differences in paw withdrawal frequencies in response to 0.07 g and 0.4 g von Frey filament stimuli among 2-, 12-, and 24-month old mice in both hind paws, although 24-month old mice exhibited a decreased tendency in response to 0.07 g von Frey filament stimulation. In addition, the mice from these three different ages showed similar paw withdrawal latencies to heat stimulation on both sides (**Figures 3E,F**).

RNA-seq and Genome-Wide Read Mapping in ACC, Hippocampus, and Amygdala of Aging Mice

To examine whether behavioral changes observed above are associated with DEGs in brain regions of aging mice, we carried out the RNA-sequencing analysis to define the gene expression profiles in the ACC, hippocampus, and amygdala from these

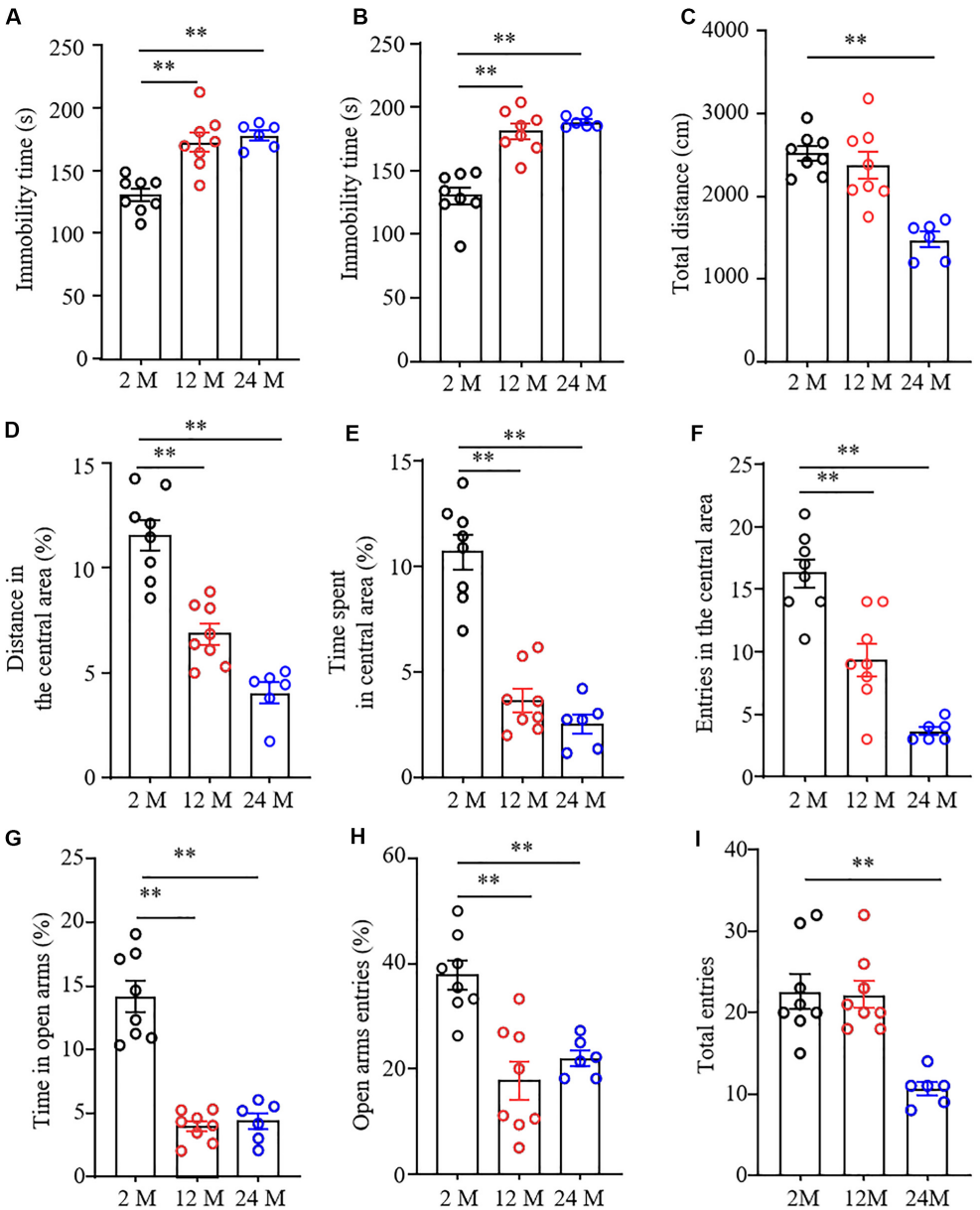

FIGURE 2 | Depression- and anxiety-like behavior in aging mice. $n = 8$ for 2- or 12-month mice, $n = 6$ for 24-month mice. Data were shown as mean ± SEM. **(A,B)** The immobility time was significantly increased in 12- and 24-month old mice in the forced swimming test (FST) **(A)** and tail suspension test (TST) **(B)** as compared to 2-month old mice. $^{**}P < 0.01$ versus 2-month old mice by one-way ANOVA with repeated measures followed by *post hoc* Tukey test. **(C)** The total distance of 24-month-old mice in the open field test was less than that of 2-month-old mice. $^{**}P < 0.01$ versus 2-month old mice by one-way ANOVA with repeated measures followed by *post hoc* Tukey test. **(D–F)** The percentage of distance in the central area **(D)**, the time spent in the central area **(E)**, and the entries in the central area **(F)** were significantly reduced in 12- and 24-month old mice in the open field test as compared to 2-month-old mice. $^{**}P < 0.01$ versus 2-month old mice by one-way ANOVA with repeated measures followed by *post hoc* Tukey test. **(G,H)** The percentages of the time spent in open arms **(G)** and open arms entries **(H)** were both significantly reduced in 12- and 24-month old mice as compared to 2-month old mice in the elevated plus maze test. $^{**}P < 0.01$ versus 2-month old mice by one-way ANOVA with repeated measures followed by *post hoc* Tukey test. **(I)** The total arm entries of 24-month old mice were less than those of 2-month old mice. $^{**}P < 0.01$ versus 2-month old mice by one-way ANOVA with repeated measures followed by *post hoc* Tukey test.

three different ages of mice. In order to visualize the collected data of DEGs, we generated the clustered heatmaps to show the changes in the genes by color intensity. As shown in **Figure 4**, compared to the gene expression profile from 2-month old mice, about 634 (510 increased and 124 decreased) and 1078 (805 increased and 273 decreased) DEGs in ACC, 453 (283 increased and 170 decreased) and 1015 (646 increased and 369 decreased) DEGs in the amygdala and 884 (485 increased and 399 decreased), and 1054 (759 increased and 295 decreased) DEGs in hippocampus were detected in 12- and 24-month old

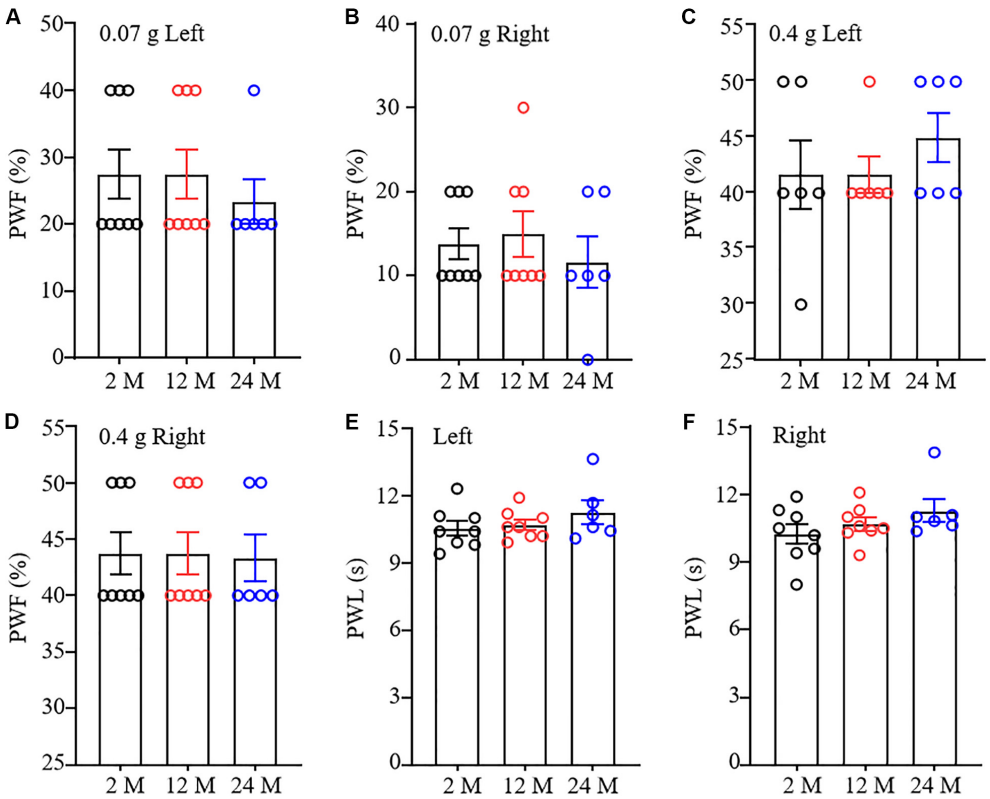

FIGURE 3 | Nociceptive response in different ages of mice. Paw withdrawal frequencies (PWF) in response to 0.07 g **(A,B)** and 0.4 g **(C,D)** von Frey filaments and paw withdrawal latency (PWL) on response to heat stimulation **(E,F)** on the left **(A,C,E)** and right **(B,D,F)** sides. n = 8 for 2- or 12-month mice, n = 6 for 24-month mice. Data were shown as mean ± SEM. One-way ANOVA with repeated measures followed by *post hoc* Tukey test.

mice, respectively. Further analysis revealed that approximately 222 (187 increased and 35 decreased) DEGs in ACC, 171 (125 increased and 46 decreased) DEGs in the amygdala and 315 (229 increased and 86 decreased) DEGs in hippocampus were seen in both 12- and 24-month old mice (**Figure 5**).

To validate the results achieved from the RNA-sequencing analysis, we randomly selected four DEGs, which were changed significantly (LFC $\geq$2.2 or $\leq$−2.2), in both 12- and 24-month old mice, for quantitative RT-PCR assay. As expected, the amount of *Igfbpl1* RNA in the hippocampus and the amount of *Ostn* RNA in the ACC were markedly reduced (**Figures 6A,B**), while the level of *Klk6* RNA in the amygdala and the level of *Defb1* RNA in the ACC were significantly increased in both 12- and 24-month old mice (**Figures 6C,D**).

Functional Enrichment Analysis of the Differentially Expressed Genes in Aging Mice

Database for Annotation, Visualization, and Integrated Discovery bioinformatics database was used to analyze the Gene Ontology analysis and categorize the DEGs based on the distinct processes. **Figure 7** showed the analysis of DEGs for biological processes. The DEGs in the ACC from both 12- and 24-month old mice were enriched highly in the immune response. The DEGs in the amygdala from 12-month old mice were enriched predominantly in immune system development, hemopoietic organ development, and hemopoiesis. The DEGs in both amygdala and hippocampus from 24-month old mice were enriched mainly in biological adhesion, cell adhesion, and immune response. The DEGs in the hippocampus from 12-month old mice were enriched largely in the regulation of cell proliferation, cell motion, cell motility, and localization of cell. We also categorized these DEGs according to their molecular functions (**Supplementary Figure 2**). The DEGs in ACC from 12-month old mice and in the hippocampus from both 12- and 24-month old mice were enriched mostly in calcium ion binding function. The DEGs in the amygdala from 24-month old mice were enriched predominantly in carbohydrate-binding function. The DEGs in the amygdala from 12-month old mice were enriched mainly in enzyme inhibitory activity, peptidase inhibitory activity, carbohydrate-binding, and endopeptidase inhibitory activity functions. The DEGs in ACC from 24-month old mice were enriched mainly in peptide binding, peptide receptor activity, cytokine activity, and hormone activity functions. Finally, we divided these DEGs according to their cellular components (**Supplementary Figure 3**). In both 12- and 24-month old mice, the DEGs in ACC were enriched predominantly in the plasma membrane and extracellular region, the DEGs in the amygdala mainly in the extracellular region

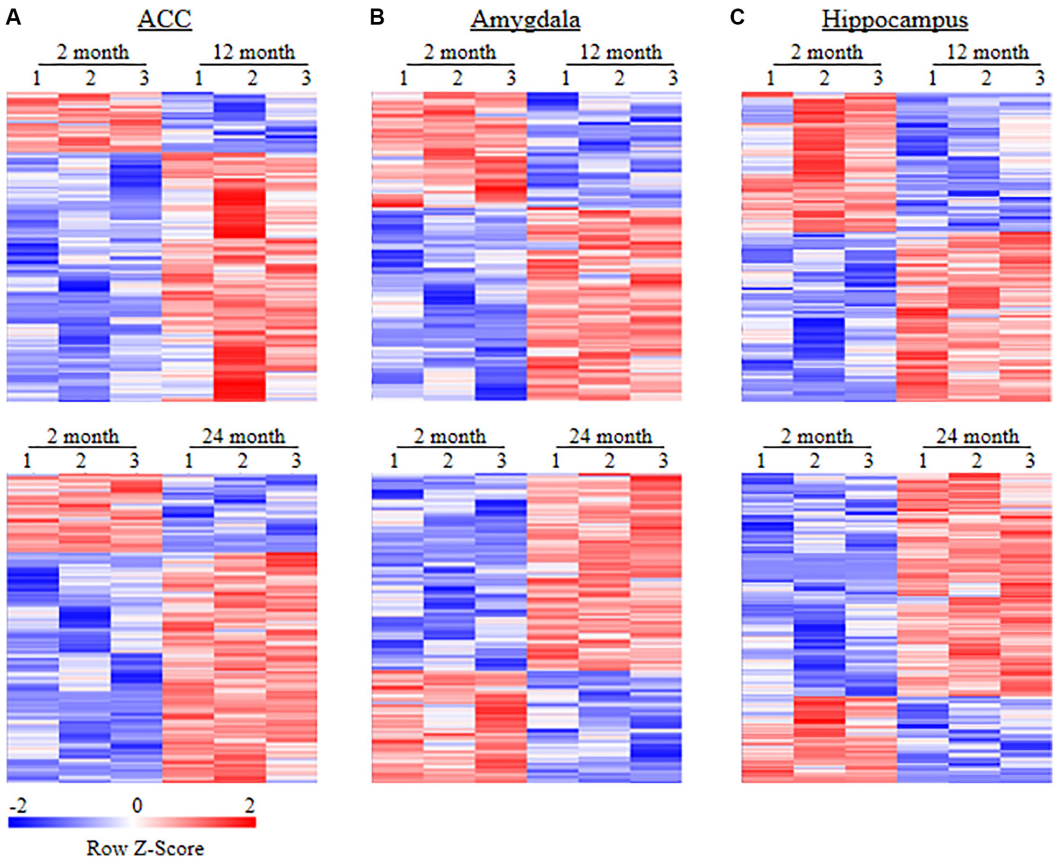

FIGURE 4 | Heatmaps of differentially expressed genes (DEGs) in the ACC **(A)**, amygdala **(B)** and hippocampus **(C)** from 2-month old mice versus 12-month old mice (top) or 24-month old mice (bottom). Colors in the heatmaps indicate the Row *Z*-score among the different data sets. High expression is shown by the red color spectrum, and low expression is shown by the blue. *N* = 3 biological repeats (3 mice)/age.

and the DEGs in hippocampus mostly in intrinsic and integral to the membrane.

Depression, Anxiety, and Cognitive Dysfunction-Associated DEGs in Aging Mice

To further analyze the relationship between DEGs and behavioral changes with aging, we used the GeneCards database and CTD database to search cognitive dysfunction, depression, and anxiety-related genes. Approximately 2000 genes were selected according to the relevance score as data sets for the subsequent analysis. Given that ACC and amygdala play an important role in the genesis of depression and anxiety and that hippocampus is a key region associated with cognitive dysfunction[8−13], we compared the depression- and anxiety-related gene data sets with the DEGs in the ACC and amygdala, and the cognitive dysfunction-related gene data set with the DEGs in the hippocampus. About 47 and 96 depression-related DEGs in the ACC and 25 and 103 depression-related DEGs in the amygdala were found in 12- and 24-month old mice, respectively (**Supplementary Material 1**). Approximately 205 and 124 anxiety-related DEGs in the ACC and 110 and 128 anxiety-related DEGs in the amygdala were seen in 12- and 24-month old mice, respectively (**Supplementary Material 1**). Finally, there were about 125 and 128 cognitive dysfunction-related DEGs in the hippocampus of 12- and 24-month old mice, respectively (**Supplementary Material 1**).

Given that the depression/anxiety-related behaviors and cognitive dysfunction were associated with inflammation, apoptosis, oxidative stress, synaptic plasticity, glutamate receptor pathway, and DNA methylation (Makhija and Karunakaran, 2013; Sheng and Erturk, 2014; Cai et al., 2015; Januar et al., 2015), we further analyzed the functions of the DEGs in the ACC, amygdala and hippocampus. All depression/anxiety/cognitive-related DEGs in three brain regions were associated with inflammation in both 12- and 24-month old mice (**Supplementary Table 2**). In addition, among the depression-related DEGs in the ACC, about 96, 87, 26, 66, and 46% from 12-month old mice and about 89, 84, 36, 67, and 92% from 24-month old mice were associated with apoptosis, oxidative stress, synaptic plasticity, glutamate receptor pathway, and DNA methylation, respectively (**Supplementary Table 2**). Among the depression-related DEGs in the amygdala, approximately 92, 80, 36, 72, and 88% from 12-month old mice and approximately 87, 88, 41, 71, and 91% from 24-month old

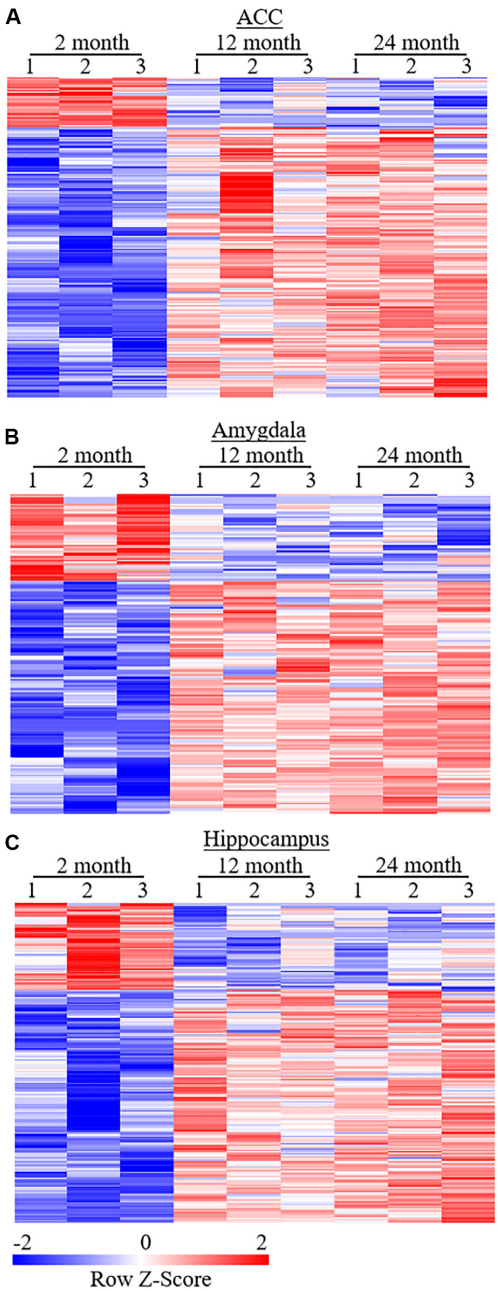

FIGURE 5 | Heatmaps of differentially expressed genes (DEGs) in the ACC **(A)**, amygdala **(B)**, and hippocampus **(C)** from both 12- and 24-month old mice versus 2-month old mice. Colors in the heatmaps indicate the Row *Z*-score among the different data sets. High expression is shown by the red color spectrum, and low expression is shown by the blue. *N* = 3 biological repeats (3 mice)/age.

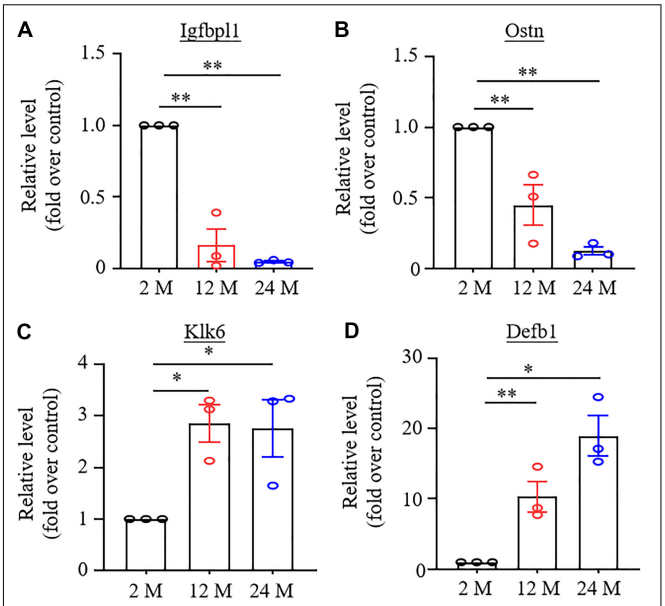

FIGURE 6 | Verification of the changes in expression of some differentially expressed genes using real-time reverse transcription polymerase chain reaction (RT-PCR) analysis. *N* = 3 biological repeats (3 mice)/age. Data were shown as mean ± SEM. **(A,B)** Expression of *Igfbpl1* mRNA in the hippocampus **(A)** and *Ostn* mRNA in ACC **(B)** were significantly reduced in both 12- and 24-month old mice as compared to 2-month old mice. $^{**}P < 0.01$ versus the corresponding 2-month old mice by one-way ANOVA with repeated measures followed by *post hoc* Tukey test. **(C,D)** Expression of *Klk6* mRNA in amygdala **(C)** and *Defb1* mRNA in ACC **(D)** were significantly increased in 12- and 24-month old mice as compared to 2-month old mice.$^{*}P < 0.05$, $^{**}P < 0.01$ versus 2-month old mice by one-way ANOVA with repeated measures followed by *post hoc* Tukey test.

mice were associated with apoptosis, oxidative stress, synaptic plasticity, glutamate receptor pathway, and DNA methylation, respectively (**Supplementary Table 2**). Among the anxiety-related DEGs in AAC, about 44, 36, and 63% from 12-month old mice and approximately 49, 42, and 60% from 24-month old mice were associated with oxidative stress, glutamate receptor pathway, and DNA methylation, respectively (**Supplementary Table 2**). Among the anxiety-related DEGs in amygdala, about 23, 30, and 56% from 12-month old mice and approximately 64, 46, and 67% from 24-month old mice were associated with oxidative stress, glutamate receptor, and DNA methylation, respectively (**Supplementary Table 2**). Among the cognitive dysfunction-related DEGs in the hippocampus, about 78, 66, 34, 53, and 75% from 12-month old mice and about 79, 70, 34, 61, and 73% from 24-month old mice were associated with apoptosis, oxidative stress, synaptic plasticity, glutamate receptor pathway, and DNA methylation, respectively (**Supplementary Table 2**).

Depression, Anxiety and Cognitive Dysfunction-Associated Genes With Differentially Expressed Isoforms in Aging Mice

We also used the same strategy to analyze the relationship between the genes with differentially expressed isoforms and behavioral changes with aging. About 525 and 507 depression-related genes in the ACC and 448 and 564 depression-related genes in the amygdala were found in 12- and 24-month old mice, respectively (**Supplementary Material 2**). Approximately 1918 and 1998 anxiety-related genes in the ACC and 1706 and

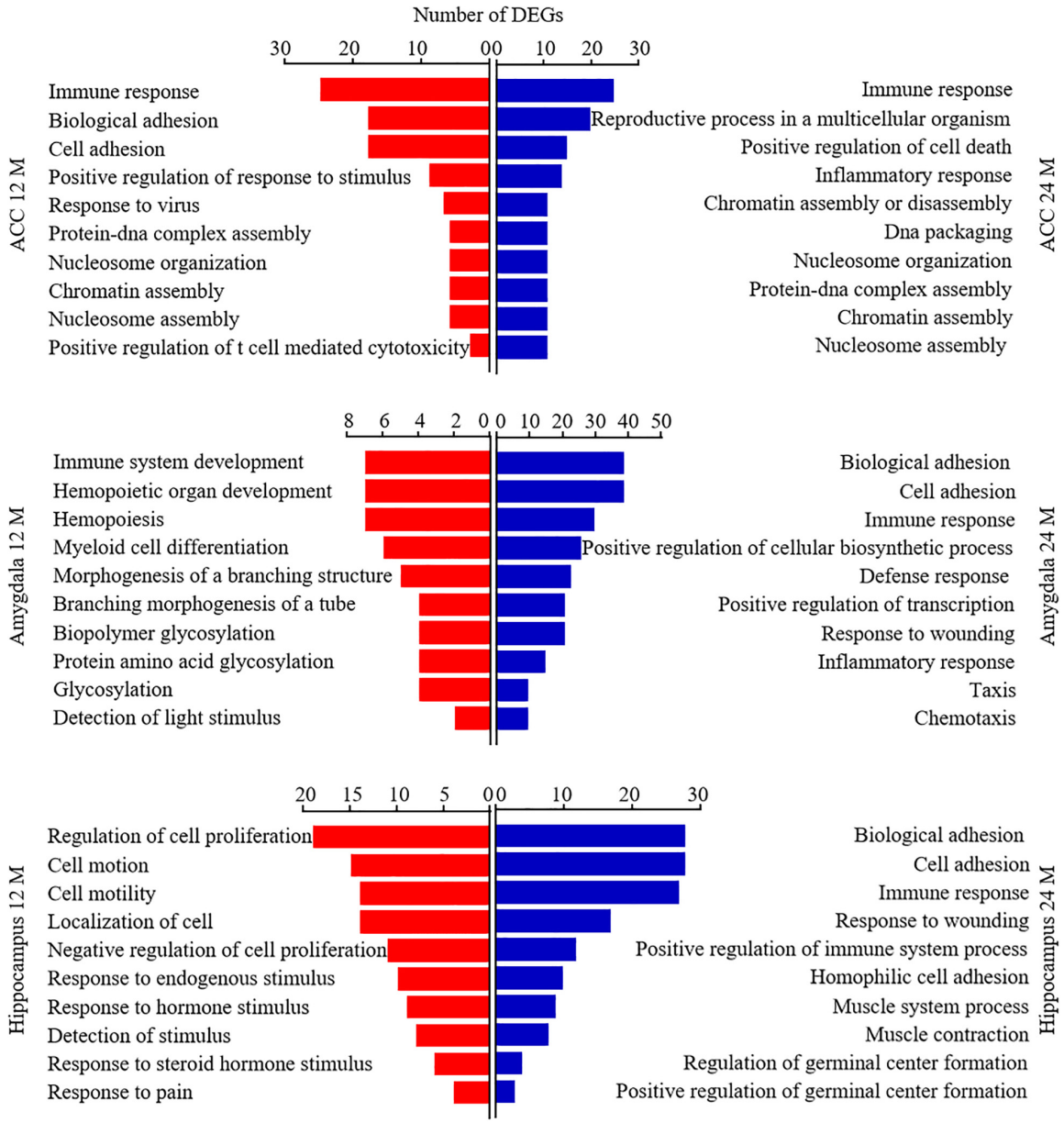

FIGURE 7 | Functional enrichment analysis of the differentially expressed genes (DEGs) in aging mice. Analysis of the Gene Ontology database showed top 10 biological processes of the DEGs in the ACC (top), amygdala (middle), and hippocampus (bottom) from 12- and 24-month old mice according to the *P*-value of biological process. The DAVID database was used to do the GO enrichment analysis. Red and blue color bars represent 12- and 24-month old mice, respectively.

1991 anxiety-related genes in the amygdala were seen in 12- and 24-month old mice, respectively (**Supplementary Material 2**). Finally, there were about 920 and 881 cognitive dysfunction-related genes in the hippocampus from 12- and 24-month old mice, respectively (**Supplementary Material 2**).

Further analysis revealed that all of these depression/anxiety/cognitive-related genes with differentially expressed isoforms in these three regions were associated with inflammation in both 12- and 24-month old mice (**Supplementary Table 3**). In addition, among the depression-related genes with differentially expressed isoforms in ACC, about 89, 80, 42, 67, and 91% from 12-month old mice and about 87, 80, 38, 64, and 87% from 24-month old mice were associated with apoptosis, oxidative stress, synaptic plasticity, glutamate receptor pathway, and DNA methylation, respectively (**Supplementary Table 3**). Among the depression-related genes with differentially expressed isoforms in amygdala, approximately 89, 80, 40, 65, and 88% from 12-month old mice and approximately 87, 78, 41, 65, and 89% from 24-month old mice were associated with apoptosis, oxidative stress, synaptic plasticity pathway, glutamate receptor pathway, and DNA methylation, respectively (**Supplementary Table 3**). Among

the anxiety-related genes with differentially expressed isoforms in AAC, about 56, 39, and 71% from 12-month old mice and approximately 55, 38, and 67% from 24-month old mice were associated with oxidative stress, glutamate receptor pathway, and DNA methylation, respectively (**Supplementary Table 3**). Among the anxiety-related genes with differentially expressed isoforms in amygdala, about 58, 38, and 69% from 12-month old mice and approximately 57, 39, and 70% from 24-month old mice were associated with oxidative stress, glutamate receptor pathway, and DNA methylation, respectively (**Supplementary Table 3**). Among the cognitive dysfunction-related genes with differentially expressed isoforms in hippocampus, about 81, 71, 35, 55, and 80% from 12-month old mice and about 82, 74, 33, 54, and 80% from 24-month old mice were associated with apoptosis, oxidative stress, synaptic plasticity, glutamate receptor pathway, and DNA methylation, respectively (**Supplementary Table 3**).

Establishing a PPI Network to Analyze Protein–Protein Interactions

To explore the relationship between the DEGs and aging pathology, we performed the PPI network analysis using the STRING database (**Figure 8**). Due to the involvement of a large number of DEGs in the network, the top 50 DEGs from each region were selected to draw the PPI network according to the connection degree. A serial of networks was generated, and the hub genes were shown in the network. Cxcl10, an important chemokine, was the central node of the network in the ACC of 12- and 24-month old mice (**Figures 8A,B**). Serpina1a, Serpinf2, Igf2, and Pomc played a crucial role in the network of the amygdala in 12-month old mice (**Figure 8C**). In contrast, Serpin family and Serpinb1b were central molecules in the network of the amygdala in 24-month old mice (**Figure 8D**). Moreover, there were 10 genes (Gcgr, Shh, Fgg, Plg, Fgf2, Cxcr4, Cxcl10, Ftcd, Edn1, Itgax, and Cxcl5) played a critical role in the network of the hippocampus of 12- and 24-month old mice (**Figures 8E,F**). The network also showed that the inflammation- and apoptosis-related genes occupied a vital position in the PPI networks (**Figure 8**).

Association of Several DEGs With Depression-, Anxiety-, or Cognitive Dysfunction-Related Behaviors in Aging Mice

Finally, we carried out the Pearson method to analyze the correlation between mRNA levels of three selected DEGs from the ACC/amygdala and the depression/anxiety-like behaviors or between mRNA levels of three selected DEGs from hippocampus and cognitive dysfunction. In the ACC, the levels of *Esr1, Crhr2,* and *Tgif1* mRNAs were positively associated with the immobile time of the forced swimming and TSTs ($r = 0.848 \sim 0.977$, $P = 0.033 \sim 0.001$; **Supplementary Table 4**). The amount of *Tshr* mRNA was negatively associated with the percentage of distance in central ($r = -0.939$, $P = 0.006$) and the entries in central ($r = -0.931$, $P = 0.007$) in OFT as well as the percentage of entries in open arms ($r = -0.968$, $P = 0.001$) and the percentage of time in open arms ($r = -0.833$, $P = 0.039$) in EPM test (**Supplementary Table 4**). The level of *Esr1* mRNA was negatively associated with the percentages of distance in central ($r = -0.856$ and $P = 0.030$) and the entries in central ($r = -0.899$, $P = 0.015$) in OFT and with the percentages of entries in open arms in EPM test ($r = -0.909$, $P = 0.012$). Interestingly, the amount of *Crhr2* mRNA was only negatively related to the entries in central in OFT ($r = -0.876$, $P = 0.022$) (**Supplementary Table 4**). In the amygdala, the immobile time of the tail suspension and FSTs was positively associated with the level of *Vdr* mRNA ($r = 0.886$, $P = 0.019$; $r = 0.872$, $P = 0.023$) and negatively related to the amount of *Kif1l* mRNA ($r = -0.850$, $P = 0.032$; $r = -0.823$, $P = 0.044$) (**Supplementary Table 4**). Unexpectedly, no significant associations were observed between *Pomc* mRNA expression and these depression-like behaviors in mice (**Supplementary Table 4**). In addition, the levels of *Bcl2l10, Serpinf2,* and *Piwil4* mRNAs displayed high positive correlations to the parameters indicated above in the OFT ($r = -0.858 \sim -0.884$ and $0.828 \sim 0.944$, $P = 0.042 \sim 0.005$; **Supplementary Table 4**). In the EPM test, the level of Serpinf2 mRNA was positively relevant to the percentage of entries ($r = 0.852$, $P = 0.031$) and the percentage of time ($r = 0.860$, $P = 0.028$) in open arms, the amount of *Bcl2l10* was negatively associated with the percentage of entries in open arms ($r = -0.852$, $P = 0.031$) and the level of *Piwil4* mRNA was unrelated to either parameter (**Supplementary Table 4**). In the hippocampus, the level of *Plg* mRNA was negatively associated with the percentages of spontaneous alternation in the Y-maze spontaneous alternation tests ($r = -0.892$, $P = 0.017$), recognition in exploring new objects 3 h ($r = -0.956$, $P = 0.003$) and 24 h ($r = -0.854$, $P = 0.030$) after training in the NOR test, and the numbers of platform crossing in the MWM tests ($r = -0.889$, $P = 0.018$) (**Supplementary Table 5**). In contrast, the level of *Dbh* mRNA was positively related to the percentages of spontaneous alternation in the Y-maze spontaneous alternation tests ($r = 0.884$, $P = 0.019$) and recognition in exploring new objects 3 h after training in the NOR test ($r = 0.855$, $P = 0.030$) (**Supplementary Table 5**). The level of *Prph* mRNA was negatively associated only with the numbers of platform crossing 1 h after training in the MWM test ($r = -0.869$, $P = 0.025$) (**Supplementary Table 5**).

DISCUSSION

Despite intensive research into the molecular and biological mechanisms of aging and aging-related diseases during past decades, aging and aging-related diseases are poorly managed by current drugs. In the present study, we used a mouse model of natural aging to mimic the different aging stages in humans to search the aging symptoms-associated genes in ACC, amygdala and hippocampus. Behavior tests were first carried out to verify depression/anxiety-like behaviors and learning, memory and cognitive impairments in the 12- and 24-month old mice. Bioinformatical analyses further showed that all DEGs and the genes with differentially expressed isoforms identified in ACC, hippocampus and amygdala of two groups of aging mice were closely related to the neuro-inflammation. Our findings demonstrate that cognitive impairment and emotional

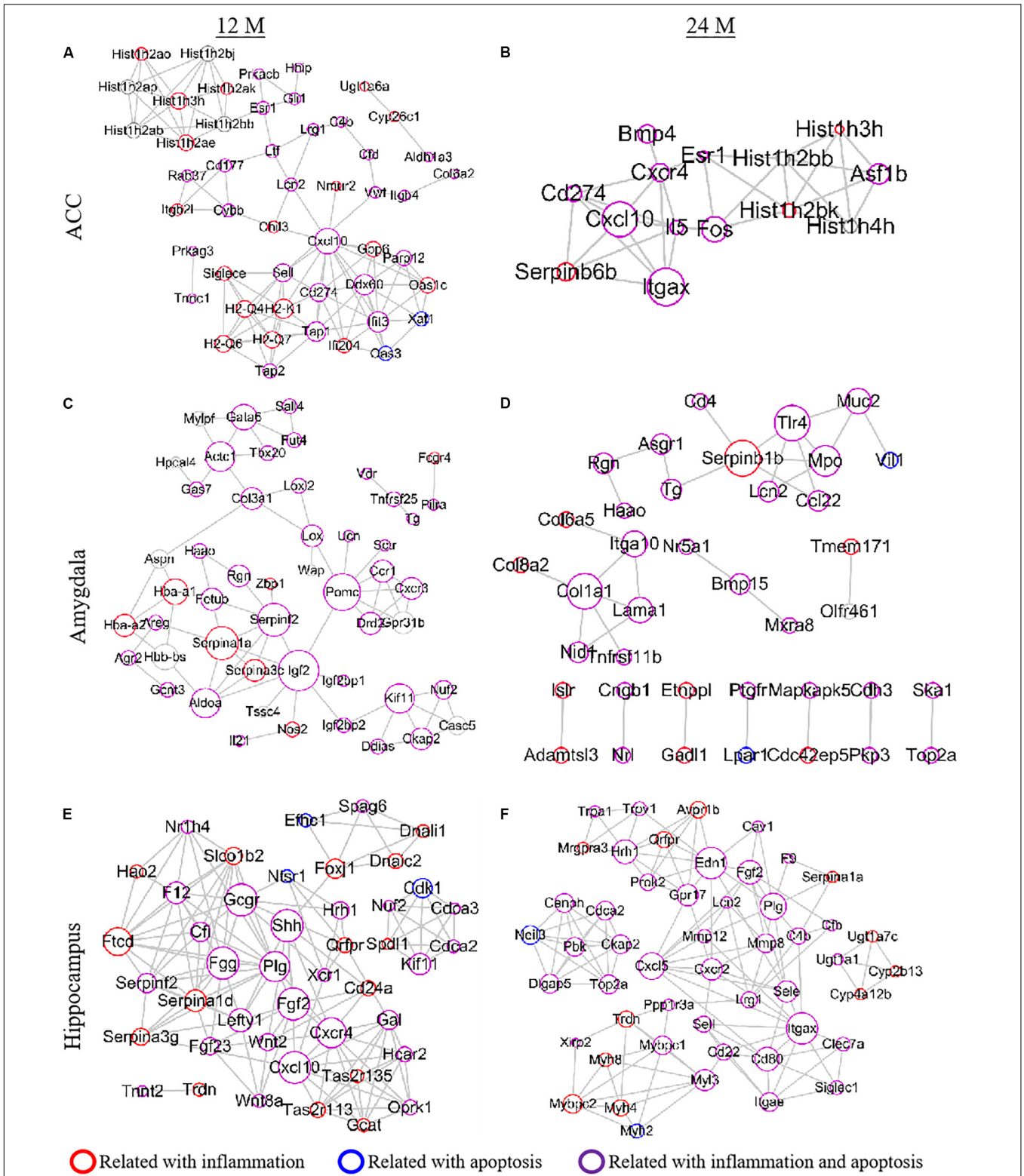

FIGURE 8 | Establishment of a PPI network to analyze protein-protein interactions. Top 50 differentially expressed genes (DEGs) were selected according to the connection degree of genes in the ACC **(A,B)**, amygdala **(C,D)**, and hippocampus **(E,F)** of 12- **(A,C,E)** and 24- **(B,D,F)** month old mice. The size of the node is determined by the connection degree. The connection degree reflects the importance of the gene in the network as it represents the number of nodes connected. The red, blue, and purple circles represent the genes related to inflammation, apoptosis and both inflammation and apoptosis, respectively.

disorders with aging are likely corresponding to the changes in neuroinflammation-related genes.

In the present study, we found that the mice of 12- and 24-month old showed the progressive loss in episodic-like memory with aging demonstrated by NOR assay. This observation was consistent with the previous reports that the object recognition memory seemed to be impaired as early as the age of 12 months (Belblidia et al., 2018). Moreover, in both groups of aged mice, spatial memory with aging was impaired evidenced by the decreased percentage of spontaneous alternation during the Y-maze test and by the prolonged escape latency, as well as the reduced numbers of crossing the position where the platform located in the training sessions during MWM test. Consistent with a previous study (Bach et al., 1999), 24-month old mice exhibited a decline in locomotor activity evaluated by the decrease in total distance traveled in OFT assay and reduced number of the entry in EPM test.

Although the mechanism of age-related emotional dysfunction remains controversial in many studies (Schulz et al., 2007; Malatynska et al., 2012), increasing evidence suggests that cognitive impairment in the aged population often accompanied by depression and anxiety disorders (Kuring et al., 2018) 52. Our findings demonstrated that the immobility time was increased in both forced swimming and TSTs in aging mice. However, in the OFT, there were no apparent changes in exploration activity between 2- and 12-month old mice, indicating depression-like behaviors mainly affected the later stage of the aging process. Anxiety-like behavior in aged mice was demonstrated by the decreased percentages of open arm entries and the time spent in open arms using EPM test, an observation in agreement with the clinical studies that memory loss and dementia were often accompanied by neuropsychiatric symptoms in abnormal aging patients (Peprah and McCormack, 2019).

The previous studies on age-related changes in pain sensitivity response are controversial (Yezierski, 2012). Several reports showed that pain perception is diminished in older people (Gibson and Helme, 2001; Gibson and Farrell, 2004), whereas other observations revealed the pain threshold increased in old age (Lautenbacher, 2012). Comprehensive reviews by EI Tumi et al. recently suggest that that old adult may be more sensitive to mechanically evoked pain but not heat-evoked pain than young adults (El Tumi et al., 2017). The present study showed no significant changes in basal sensitivities in response to evoked mechanical and thermal stimuli in both 12- and 24-month old mice compared to 2-month old mice, which is in line with the previous reports in the animals (Fahlstrom et al., 2012; Shoji et al., 2016; Millecamps et al., 2019; Shoji and Miyakawa, 2019). The reasons of why preclinical studies are inconsistent with clinical observations are unclear, but might be due to the inconsistent stages of the aging process between humans and animals as well as the limited measurement methods, parameters, and dimensions in animal studies.

Despite more and more attention focused on aging and age-related disorders in clinical and preclinical investigations, the underlying mechanisms remain unclear. To gain insight into the aging process and provide the potential targets, gene expression profile changes from aging mice were detected in the present study. We identified about 634 and 1078 DEGs in ACC, 453 and 1015 DEGs in the amygdala, 884 and 1054 DEGs in the hippocampus in the 12- and 24-month old mice, respectively. We also identified many genes with differentially expressed isoforms in these three brain regions in the 12- and 24-month old mice. Further function analysis of DEGs and the genes with differentially expressed isoforms revealed that many of them in the ACC and amygdala were mapped to depression- and anxiety-related genes, respectively and that a lot of them in hippocampus were mapped to cognitive dysfunction-related genes from both 12- and 24-month old mice. All of these mapped DEGs and the genes with differentially expressed isoforms were closely related to neuroinflammation. Some of them were associated with apoptosis, oxidative stress, synaptic plasticity, glutamate receptor pathway or DNA methylation. We found that Cnr2 and Tlr4 genes related to neuroinflammation were robustly upregulated in the ACC and amygdala of aging mice, suggesting that these two genes play an important role in the genesis of depression and anxiety during aging. This conclusion is strongly supported by the previous studies that showed that knockout of Cnr2 in the brain could attenuate psychomotor, depression- and anxiety-like behaviors in mice (Liu Q.R. et al., 2017) and that blocking Tlr4 could reduce the anxiety- and depression-like behaviors (Strekalova et al., 2015; Zhang et al., 2020). In addition, we also found that the other two neuroinflammation-related genes Fgf2 and Cbln1 were differentially expressed in the hippocampus of aging mice, both of which have been demonstrated to affect fear conditioning and spatial memory processes in aged animals (Hirai et al., 2005).

It should be pointed out that present study has several limitations to be considered. Firstly, although we reported significant correlations of several selected DEGs in AAC, amygdala and hippocampus with depression-, anxiety-, or cognitive dysfunction-like behaviors, these correlations do not imply causation. Thus, we should be very careful for the interpretation of our data and perform more stringent researches to examine the roles of these candidate genes in depression, anxiety or cognitive dysfunction. Secondly, the expressional levels of some selected DEGs were not significantly associated with some parameters in behavioral tests or had no correlations with depression-, anxiety-, or cognitive dysfunction-like behaviors. These non-associations/non-correlations may be attributed to low number of the samples per group (n = 3 repeats/group). Increasing number of the samples per group may improve correlation efficient. Thirdly, our research on depression, anxiety and cognitive dysfunction in aging focused on male mice. There have been few studies on female mice so far. However, gender-specific effect of DEGs on depression, anxiety and cognitive dysfunction in aging could exist and remains to be addressed in the future.

CONCLUSION

In conclusion, we demonstrated that progressive memory impairment and emotional disorders with aging were relevant

to the neuroinflammation-related DEGs and the genes with differentially expressed isoforms in ACC, amygdala and hippocampus. Our findings may uncover a new layer of aging-related transcriptional regulation and be helpful for the drug development for management of aging and age-related disorders.

DATA AVAILABILITY STATEMENT

All sequencing data are available in NCBI database (accession number: SRP271007).

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of Zhengzhou University.

AUTHOR CONTRIBUTIONS

YA and Y-XT conceived the project, supervised all experiments, and edited the draft. ML, SS, JC, XM, WZ, JY, Y-XT, and YA assisted with experimental design. ML, SS, and XM took care of feeding and maintenance of old mice. ML, SS, and WC carried out behavioral tests. ML performed tissue collection and RT-PCR assay. ML, SG, and YX analyzed the data and wrote the draft of manuscript. All authors read and discussed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00814/full#supplementary-material

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REVIEW
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Early Life Stress Induced DNA Methylation of Monoamine Oxidases Leads to Depressive-Like Behavior

Qiuyue Xu[1†], Mingchen Jiang[1†], Simeng Gu[2†], Fushun Wang[3*] and Bin Yuan[1,4]*

[1] School of Medicine, Nanjing University of Chinese Medicine, Nanjing, China, [2] Department of Psychology, Jiangsu University Medical School, Zhenjiang, China, [3] Institute of Brain and Psychological Sciences, Sichuan Normal University, Chengdu, China, [4] Jiangsu Key Laboratory of Pediatric Respiratory Disease, Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, China

Major depressive disorder (MDD) is coming to be the regarded as one of the leading causes for human disabilities. Due to its complicated pathological process, the etiology is still unclear and the treatment is still targeting at the monoamine neurotransmitters. Early life stress has been known as a major cause for MDD, but how early life stress affects adult monoaminergic activity is not clear either. Recently, DNA methylation is considered to be the key mechanism of epigenetics and might play a role in early life stress induced mental illness. DNA methylation is an enzymatic covalent modification of DNA, has been one of the main epigenetic mechanisms investigated. The metabolic enzyme for the monoamine neurotransmitters, monoamine oxidases A/B (*MAO A/MAO B*) are the prime candidates for the investigation into the role of DNA methylation in mental disorders. In this review, we will review recent advances about the structure and physiological function of monoamine oxidases (MAO), brief narrative other factors include stress induced changes, early life stress, perinatal depression (PD) relationship with other epigenetic changes, such as DNA methylation, microRNA (miRNA). This review will shed light on the epigenetic changes involved in MDD, which may provide potential targets for future therapeutics in depression pathogenesis.

Keywords: monoamine oxidase, major depressive disorder, epigenetics, DNA methylation, early life stress (ELS)

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***Correspondence:**
Simeng Gu
gsm_2007@126.com;
398989312@qq.com
Fushun Wang
13814541138@163.com

† These authors have contributed equally to this work

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INTRODUCTION

Major depressive disorder (MDD) is a major health problem and one of the leading causes of disability worldwide, and has an estimated lifetime prevalence of 16% (Gu et al., 2016). Due to its complexity, the pathology of MDD is still unclear. Recently, many studies have found that early life stress can induce long term changes in neural changes or behavioral changes. However, even though many theories suggest that the early life stress can affect the adult emotions and behaviors in a long

Abbreviations: 5-HT, 5-hydroxytryptamine; 5-HT1A, serotonin 1-A receptor; ALKBH5, alkB homolog 5; AVP, arginine vasopressin; BDNF, brain-derived neurotrophic factor; CRMP2, collapsing response mediator protein 2; CUMS, chronic unpredictable mild stress; DNAm, DNA methylation; ELS, early life stress; FKBP5, FK506-binding protein 5; HSD11B2, Type-2 11b-hydroxysteroid dehydrogenase; m^6A, N^6-methyladenosine; MAO, monoamine oxidases; MDD, major depressive disorder; miRNAs, microRNAs; MORC1, microrchidia family CW-type zinc-finger 1; ncRNAs, non-coding RNAs; NR3C1, nuclear receptor subfamily 3 group C member 1; NR3C2, nuclear receptor subfamily 3 group C member 2; NSCs, neural stem cells; OXTR, oxytocin receptor; PD, perinatal depression; PPD, postpartum depression; SLC6A4, serotonin transporter-encoding gene; Tacr2, tachykinin receptor 2.

run, but the underlying neural mechanism is far from clear. Since the etiology of MDD includes the interaction between genes and the environment, epigenetics is important for predicting utility and treatment monitoring (Webb et al., 2020). Stressful life experiences, especially early life stress, might carry out epigenetic modification of these risk genes via DNA methylation and microRNAs (miRNAs) regulation, and the expression of these genes will have long-lasting effects, which will lead to changes in brain structure and function (Pishva et al., 2017; Ding and Dai, 2019). In addition, the epigenetic differences may affect treatment response (Webb et al., 2020), however, the epigenetic mechanism of antidepressant drugs is not fully understood.

The monoamine neuromodulators have also been related to affective disorders ever since 50s in the last century and drugs targeting monoamine neuromodulators have been considered to be the first choice of treatment for these mental diseases. In addition, the monoamine neuromodulators have recently been identified as the primary neural substrates for three core affects: Dopamine-reward, Norepinephrine-stress, serotonin-punishment (Gu et al., 2019). They work together to make different basic emotions, like the three primary colors. The DA system has been proved to be involved in reward (joy), the NE has been related to the "fight or flight" (fear and anger) responses at stressful events, and the 5-HT system seems to be related to punishment (sad) (Wang et al., 2020). And dysfunctions of the monoamine system are involved in many mental disorders such as depression, post-traumatic disorders, anxiety, and attention-deficit hyperactivity disorder. Indeed, some early life events indeed induce epigenetic changes for many monoamine receptors or transporters, such as methylation DNA of MAO, or through miRNA changes. These changes would induce dysfunction of monoaminergic systems which are related to affective disorders.

Monoamine oxidase (MAO) is a major enzyme that modulates the metabolism of monoamine transmitters, including dopamine, 5-hydroxytryptamine (5-HT), norepinephrine. MAO has neurobiological origins and functions that is a potential therapeutic target in neuronal drug therapy. The *MAO A/B* genes are located on the X-chromosome (Xp11. 23) and comprise 15 exons with identical intron-exon organization, which suggests that they derived from the same ancestral gene (Shih et al., 1999). MAO regulates the levels of monoamine neurotransmitters in the brain thereby affecting signal transduction pathway and gene expression to regulate brain function, finally affecting many functions of neurons (Naoi et al., 2018). MAO catalyzed the major inactivation pathway for the monoamine neurotransmitters (Youdim and Bakhle, 2006). It is shown that *MAO A/B* act as mediators or repressors of gene expression, respectively (Inaba-Hasegawa et al., 2017; Naoi et al., 2018). *MAO A* activity fluctuates under the influence of genetic environmental factors, modulates the response of neurons to stimuli, and affects emotional and behavior activity. *MAO B* inhibitors selegiline and rasagiline can increase the expression of anti-apoptotic Bcl-2 and pro-survival neurotrophic factors in human neuroblastoma SH-SY5Y and glioblastoma U118MG cell lines Protect neurons.

Monoamine oxidase inhibitors were the first antidepressants to be developed in last century. Monoamine oxidase inhibitors increase the levels of norepinephrine, 5-HT, and dopamine by inhibiting an enzyme called monoamine oxidase (Saka, 2017). After the initial "golden age," MAO inhibitors are currently used as third-line antidepressants (selective *MAO A* inhibitors) or clinically included as adjuvants for neurodegenerative diseases (selective *MAO B* inhibitor). However, because of its key role in regulating synapse function and monoamine metabolism, research in this field are increasing (Carradori et al., 2018). In this review, we briefly review the existing physiological data of MAO, summarize the interaction between *MAO A* and *MAO B* gene methylation and environmental factors, and discuss the pathogenesis of depression in different causes. MAO methylation mode can be used as a depression peripheral biomarker of risk and treatment responses, which might provide knowledge for future prevention and personalized treatment methods. In addition, we will provide some experiment evidence and discuss the latest advances in epigenetics and depression research. These evidences suggest that chronic unpredictable mild stress, early life stress, and perinatal stress-induced MDD vulnerability are related. Finally, we speculate future works that is needed to better understand the harmful effects of stress on MDD risk.

Structure and Physiological Function of MAO

Monoamine oxidases (MAOs) are mitochondrial outer membrane flavoenzymes, and are composed of 527 and 520 amino acid residues, with their molecular weights being approximately 59,700 and 58,800 for MAO A and MAOB respectively. The active forms of the isozymes *MAO A* and *MAO B* are homodimers, which are determined by their complementary DNA structures. These two isozymes are distinguished by tissue and cell distribution, substrate selectivity, inhibitor sensitivity, and separate codes (Bach et al., 1988; Ramsay, 2016). The first crystal structure of MAOs was solved in 2002 (Iacovino et al., 2018). In order to easily describe the relationship between the structure and function of this enzyme, we can use the PyMOL software, which is a molecular visualization system, to watch the structure of human *MAO A* and *MAO B*. The coordinates and the structural factors have been deposited in the Protein Data Bank, www.pdb.org (*MAO A*: PDB ID codes 2Z5X and *MAO B*: PDB code 1GOS) (Binda et al., 2004; Son et al., 2008), At the same time, we can observe the stereo view of the superposed structures of the human *MAO A* and human *MAO B* (**Figure 1A**).

It was found that MAO has a flavin adenine dinucleotide binding site, which is inclined to be hydrophobic aliphatic and aromatic. Therefore, the different amino acid residue binding sites in these two enzymes play a decisive role in the choice of substrates and inhibitory drugs. They catalyze the oxidative deamination of various neurotransmitters. Its main role is to catalyze the monoamines in the cell, so that the monoamines are oxidized to produce deamination. MAO acts on primary amines and their methylated secondary and tertiary amines, as well as long-chain diamines (Tipton, 2018; Sánchez-Rodríguez et al., 2020). The amino acid sequence of *MAO A* and *MAO B*

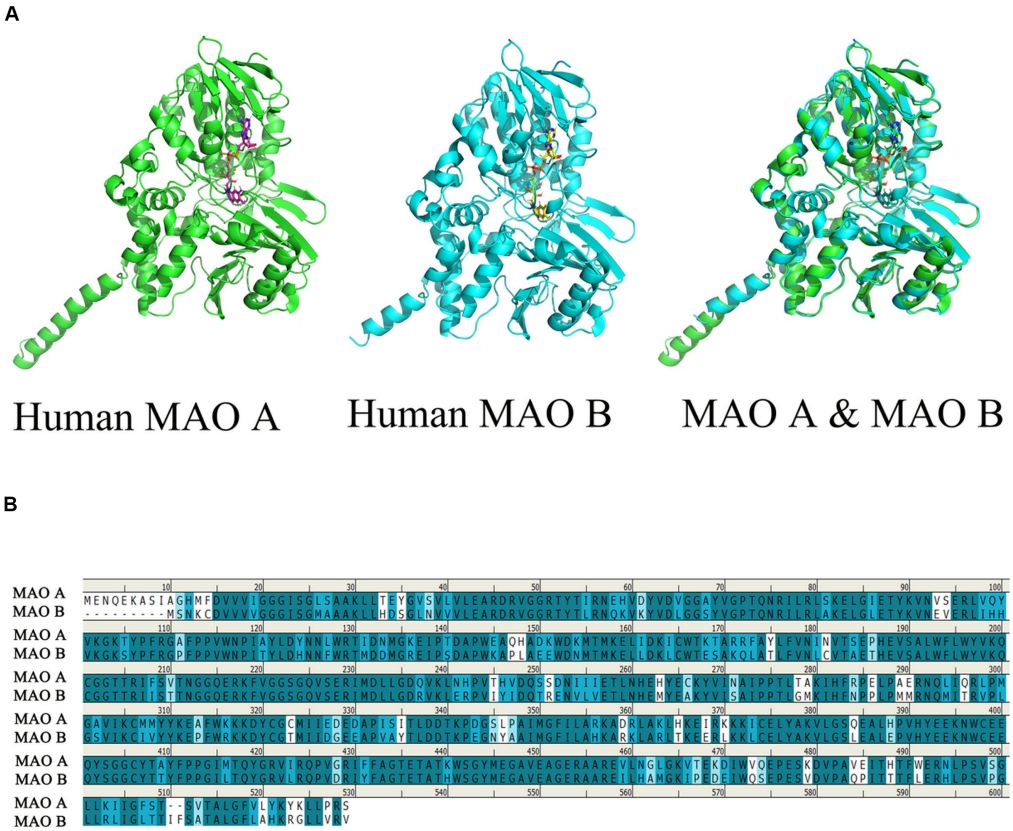

FIGURE 1 | Comparison of the structure of human *MAO A* and human *MAO B*. The picture is generated by PyMOL (Son et al., 2008). Use clustalw in MEGA software to perform multiple sequence alignment, generate alignment result files, and import the aligned sequences into geneDoc software for visualization (Boratyn et al., 2019). **(A)** Observe the overlapping structure of human *MAO A* and human *MAO B* in three-dimensional space. The same part between the two structures is shown in cyan. **(B)** Amino acid sequence identity comparison, blue is the sequence with higher similarity, the darker the color, the higher the similarity, the overall sequence identity between *MAO A* and *MAO B* is about 71.1%.

can be up to 71.1% identical (**Figure 1**), although each enzyme has unique substrate and inhibitor specificity, *MAO A* firstly oxidizes serotonin or 5-HT and noradrenaline, whereas *MAO B* preferentially oxidizes beta-phenylethylamine (Grimsby et al., 1997). Base on the structure and physiological function of MAO, the unique position of MAO in modulating the function of a diverse series of specific neurotransmitters in association with various conditions.

MAO DNA Methylation in Depression

DNA methylation is an epigenetic mechanism used by cells to regulate gene expression. There are many studies that reported the correlation between DNA methylation and human brain structure/function, and these studies suggest the DNA methylation can induce the following diseases: neurodevelopmental and neurodevelopmental disorders; major depression and psychosis (Wheater et al., 2020). In MDD, increased *MAO A* expression and decreased serotonin and norepinephrine brain levels are considered to be the major causative factors. Functional polymorphisms of the *MAO A* gene and genes in the serotonin signaling pathway are associated with depression. Depression in females may result from a dysregulated epigenetic programming of *MAO A*. Melas et al., have shown that females depression is related to hypomethylation in the first exon region of the *MAO A* gene. A small-scale (n = 44) replication study of *MAO A* methylation confirmed that female subjects with a history of depression had a hypomethylated *MAO A* compared with the control group, and the study also shows that females are hypermethylated in the same area compared with that of males (Melas and Forsell, 2015). They also found that female depressive patients showed significantly decreased methylation at ten methylated sites (CPGs) representing parts of exon I and intron I of the *MAO A* gene, compared with age-matched healthy female controls, discerned (n = 82–92) (Melas et al., 2013). DNA methylation levels have a major impact on depression. Interestingly, there are relatively few studies on DNA methylation of the gene encoding *MAO B*. Nevertheless, there is evidence for depression and changes in *MAO B* methylation patterns. In a sample of N = 199 single-egg twins without major depression, the initial association between a *MAO B* promoter CpG site and depressive symptoms could not be corrected in multiple tests (Peng et al., 2018). In fact, different environmental stress factors will affect the DNAm of different CpGs, which will affect the phenotype of depression (**Table 1**).

Chronic Unpredictable Mild Stress Affects Methylation

Stress is an evolutionarily adaptive response to deal with situations that impact threat to the organism and require rapid "flight or fight" responses (Wang et al., 2017). It is essential for survival and benefits to all lives, however, overwhelming stress is considered to one of the main risk factors for the development of many emotional disorders such and anxiety, depression. For example, the onset of major depression are often correlated with stressful events in previous lives, many studies reported significant correlation between the onset of major depression and the number of life altering events in the previous 3 months (Wang et al., 2017). Sometimes the previous stress happens very long ago, for example, early life stress can induce emotional depression in adult lives. This means stress can induce long term changes in the body to induce emotional disorders. Long-term or excessive stress, especially the stress generated in early life, is considered to be a high-risk environmental factor that induces various mental diseases such as depression. Elucidating the underlying molecular processes of stress-related transcriptional responses is essential for understanding the development process of stress-related mental illnesses.

It was found that patients with depressive disorder had lower *MAO A* methylation than healthy controls (Ziegler et al., 2016). Although this is not enough to explain that insufficient *MAO A* methylation can lead to depression, insufficient *MAO A* methylation may become a risk sign of mental disorders. In other words, insufficient *MAO A* methylation may be a cause of depression caused by stress. Similar to DNA modification, there are a series of different covalent modifications on RNA nucleotides encoding exotranscripts, which form gene expression by regulating RNA stability, translation and non-coding transcription functions. RNA modification, second only to epigenetic mechanisms, may represent an undescribed level of transcriptional regulation, which is highly relevant to psychiatry like MDD. Dominissini et al. found N^6-methyladenosine (m^6A) is the most abundant internal mRNA modification, and it is present in the entire transcriptome, at least in one-fourth of the RNA (Dominissini et al., 2012; Li Y. et al., 2020). It is found that single point mutations or expression mutations in the gene encoding neuronal glycoprotein m^6A are associated with mental illness, and m^6A modified genes may carry MDD risks (Du et al., 2015; Garcia et al., 2017). Researchers have used m^6A/m sequencing (m^6A/m-seq) and absolute quantification of transcript-specific methylation levels and found that m^6A/m methylation in the cortex is overrepresented in genes involved in synaptic and neuronal regulation. m^6A/m-RNA Methylation may be related to stress-induced mental illness (Engel et al., 2018).

Methylated RNA immunoprecipitation sequence analysis in the peripheral blood found that circSTAG1 is significantly reduced in chronic unpredictable mild stress (CUMS) mice and MDD patients. On the contrary, overexpression of circSTAG1 induced a positive effect on CUMS-induced astrocyte dysfunction and depression-like behavior (Huang et al., 2020). With the same detection method, the methylation level of the serotonin 1-A receptor (*5-HT1A*) promoter was found to be closely related to mRNA transcription and protein expression, the presence of stress increased the level of *5-HT1A* mRNA in the prefrontal cortex of CUMS mice by 50%. The methylation of the -681CpG site might be the main cause of *5-HT1A* transcription induced by stress (Le François et al., 2015; Albert et al., 2019).

Similarly, there are studies showing that the DNA methylation level of the collapsing response mediator protein 2 (*CRMP2*) in the hippocampus of the CUMS group was significantly higher than that of the control group, but these changes were not observed in the prefrontal cortex of CUMS rats. This indicates that the changes in *CRMP2* expression in the hippocampus and prefrontal cortex are related to the pathogenesis of depression. In addition, the results also show regional differences in the regulation of DNA methylation in the *CRMP2* promoter between the hippocampus and the prefrontal cortex during the development of depression (Xiang et al., 2020). DNA methylation is a dynamic tissue-specific event that may play an important role in the persistent and recurrent nature of depression.

Changes Caused by Early Life Stress

Early life stress is a critical causing factor for many types of mental disorders, such as depression, anxiety, posttraumatic stress (Wang et al., 2020). Many studies have found that early life stress can induce long term changes in neural changes or behavioral changes. However, even though many theories suggest that the early life stress can affect the adult emotions and

TABLE 1 | Epigenetic changes depression: related gene and expression.

Depression	DNA methylation site	Gene expression/affect	References
CUMS	− 681 CpG	5-HT1A	Le François et al., 2015; Zurawek et al., 2019
	− 694 to −105 CpG	CRMP2	Li W. et al., 2020; Xiang et al., 2020
	− 863 to −732 CpG	Tacr2	Micale et al., 2008; Xiang et al., 2019
ELS	Hypothalamic neurons CpG	NR3C1	Bockmühl et al., 2015; Holmes et al., 2019
	chromosome 6p21.31	FKBP5	Harms et al., 2017; Tozzi et al., 2018
	CRCh37/hg19	MORC1	Nieratschker et al., 2014; Thomas et al., 2020
PD	HSD11B2	cortisol	Appleton et al., 2015; Seth et al., 2015
	−?934 CpG	OXTR	Maud et al., 2018; Tops et al., 2019
	Chromosome 3	AVP	King et al., 2017; Solomonova et al., 2019
PPD	8810078 and 8810069 CpG	Antenatal serum estradiol	Kimmel et al., 2016; Osborne et al., 2016
	− 22 to −23 CpG	NR3C1	Oberlander et al., 2008; Murgatroyd et al., 2015

behaviors in a long run, but the underlying neural mechanism is far from clear. Exposure to stress during critical periods in development can have severe long-term neural changes (Wang et al., 2020). Even though many neural and hormone changes have been suggested to underlie the changes for adult depression, but how early life stress affect adult affective disorders is still not clear. Recent epigenetic studies offer some answers for this process (**Figure 2**), indeed some early life events really induce epigenetic changes for many neuromodulator receptors or transporters, such as methylation DNA of MAO.

The normal development of the brain requires strict regulation of proliferation and differentiation of neural stem cells (NSCs), thereby ensuring specific number of neurons to be generated at a specific time and at a specific location (Tang et al., 2019). DNA demethylation of CpG dinucleotide sites play an important role in the cell fate characteristics of NSCs (He et al., 2020). In mouse cortex, astrocytes are mainly produced between echovirus 16 and the postpartum stage (Sauvageot and Stiles, 2002; Juliandi et al., 2010). The methylation of the Gfap promoter, a typical astrocyte marker, is anti-correlated with the expression of Gfap as same as astrocytes (Takizawa et al., 2001). DNA methylation inhibits the activation of glial production by hypomethylation of astrocyte lineage gene promoters. DNA methylation is one of the major epigenetic mechanisms that can regulate the cell fate of NSCs and control the order of neuron and glial production (Feng et al., 2007). If problems occur in the early regulatory process, the development of the brain will be affected and may lead to brain diseases.

Early life stress can "damage" the brain, leading to the possibility of developing depression later in life through epigenetic mechanisms. Early and adult stress exposure is related to epigenetic changes in genes related to mood regulation, for example, participation Genes that regulate hypothalamic-pituitary-adrenal axis activity (*NR3C1*) or genes responsible for serotonin transporter (5-HTT)-encoding gene SLC6A4 (Wang et al., 2020). Data from epigenetic studies indicate that the mechanism of action of certain antidepressants (such as fluoxetine and escitalopram) or mood stabilizers (such as valproic acid) are at least partially related to epigenetic processes (Chmielewska et al., 2019). The association between early life stress and depression is controlled by genetic risk factors, including serotonin transporter, brain derived neurotrophic factor, glucocorticoid receptor, FK506-binding protein 5 and corticotropin releasing hormone receptor 1 polymorphisms. At the same time, early life pressures will make epigenetic modifications to these risk genes through DNA methylation and miRNA regulation, which will have long-term effects on the expression of these genes, and cause changes in brain structure and function, and eventually increase Genetic susceptibility (Ding and Dai, 2019).

In recent years, the emergence of small non-coding RNAs (ncRNAs) as large controllers of gene expression has attracted attention due to their roles in various disease processes (Wen et al., 2020). Among various ncRNAs, miRNA is mostly studied, and has been regarded as the main regulator of neuroplasticity and brain function. miRNA has been proved to play a role in the maladaptive processes associated with ELS in puberty and adulthood (Wu et al., 2019). Postpartum ELS is associated with abnormal miRNA expression and function, and these processes are essential for the development of depression and suicidal behavior (Allen and Dwivedi, 2020).

Sex Hormone and Depression

Recent studies have shown that female depression may be caused by gene and child-adversity interaction and/or *MAO A* epigenetic programming disorder (Melas et al., 2013). Observation from epidemiological data shows that the incidence of MDD in women is 2.5–3 times that of men. During puberty, the incidence of depression increases. After puberty, girls show a steeper upward trend than boys. It is also worth noting that women also experience many emotional disorders during the perinatal or menopausal period. The timing of the onset of this sex bias suggests that sex hormones contribute to depression and anxiety during puberty (Gu et al., 2018). About 20% of new mothers will develop perinatal depression (PD), which is one of the most common medical complications during and after pregnancy. The depression has short-term or long-term negative effects on mothers, children and their families. Current studies have reported many causes of PD, including genetic, epigenetic, environmental, socioeconomic and psychosocial risk factors

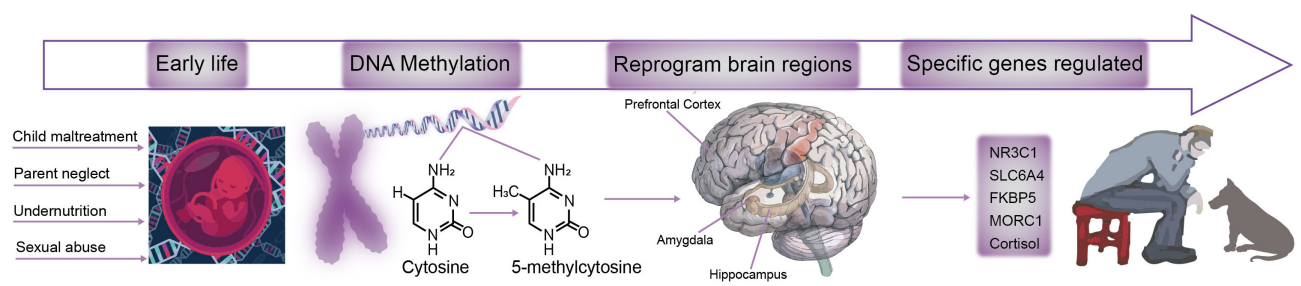

FIGURE 2 | A model for the mechanism of DAN methylation affects brain function. During development, the brain is sensitive to environmental changes, such as child maltreatment, parent neglect, less nutrition, or sexual abuse. ELS induces changes in the DNA methylation-5-methylcytosine pattern, which leads to brain damages in hippocampus, prefrontal cortex, amygdala and other brain regions are reprogrammed. As a result, specific genes such as *NR3C1*, *SLC6A4*, *FKBP5*, *MORC1*, and Cortisol are impaired, and ultimately lead to an increased risk of depression and other stress-related diseases.

(Hoffman, 2020). Pregnancy will induce epigenetic and other downstream changes in the maternal Oxytocin-system, which may be mediated by the action of steroid hormones. Oxytocin receptor (OXTR), a key regulator of stress and anxiety, is also affected by gonadal hormones and psychosocial risk factors. Hormonal changes cause changes in the DNA methylastion of the oxytocin gene locus promoter throughout pregnancy, affecting maternal behavior (Toepfer et al., 2019). *TTC9B* and *HP1BP3* DNA methylation during antenatal time are associated with the changes of estradiol and allopregnanolone over the course of pregnancy (Osborne et al., 2016). Estradiol levels and MAO DNA methylation also exhibited a significant interaction to associate with the ratio of allopregnanolone to progesterone (Kimmel et al., 2016).Cumulatively, the specific increased sensitivity of epigenetic reprogramming of postpartum depression (PPD) on MAO genes is confirmed, and it is indicated that epigenetic variation may be an important mediator of mood-related steroid production.

Many recent studies suggested that depression may be a reflection of chronic maternal stress and may result from glucocorticoids, all of which are related to the etiology of psychotic diseases (Stein et al., 2014).The association between stress during pregnancy and epigenetic modification of offspring DNA and the methylation of offspring DNA is also reproted (Nowak et al., 2020). Two genes are reported to be involved in stress response regulation: nuclear receptor subfamily 3 group C member 1 and 2 (*NR3C1* and *NR3C2*). DNA methylation of *NR3C1* and *NR3C2* were measured in placental and infant buccal samples, and it is found that maternal early pregnancy depressive disorder and symptoms were associated with lower DNA methylation at *NR3C2* CpG_24 in placental tissue. The changes in *NR3C2* DNA methylation levels seem to be affected by infant cortisol, which indicates that DNA methylation intervention in infancy may come from maternal hormones, and the main effect is *NR3C2* (Galbally et al., 2020). However, in childhood-adversity subtypes may differentially impact DNA methylation at *NR3C1*, baseline *MAO A* variations may affect the extent of *NR3C1* methylation (Melas et al., 2013). *MAO A* was also suggested to acts simultaneously with *NR3C2* methylation. Treatments that improve maternal depressive symptoms can reduce children's overall DNA methylation, increase the thickness of the cortex, and reduce the cross-section of white matter fiber bundles in areas involved in cognitive function and stress response (Bleker et al., 2020).

miRNA Relationship With DNA Methylation

Epigenetics refers to processes that affect gene expression and translation, including DNA methylation and miRNA and histone modifications. The methylation of RNA and DNA, in the form of m^6A and 5-methylcytosine plays a vital role in various biological processes (Chen et al., 2016). DNA methylation can affect m^6A modification by modulating the expression of m6A demethylase gene, and m^6A demethylase feedback regulates DNA methylation, thus established a molecular relationship between 5-methylcytosine DNA methylation and m^6A mRNA methylation during fruit ripening (Zhou et al., 2019). Further studies with human diseases found mutation frequencies of m^6A and 5-methylcytosine regulators were increased in depression. Interestingly, the m^6A and 5-methylcytosine regulators show a considerable level of mutation frequency in depressive patients and these two regulators also happen together (Chen et al., 2020).

DNA methylation, modification of histone and chromatin structures, and the function of ncRNAs are the core regulators for specific patterns of gene expression. Epigenetic modifiers, especially microRNAs (miRNAs), are attracting more scientific efforts because of their role in stress sensitivity after early stress. Among them, potential genetic and environmental risk factors may drive abnormal epigenetic changes to target stress response pathways, which are related to neuronal plasticity and major depression. miRNA is ∼22 nt RNA that can guide post-transcriptional inhibition of mRNA targets in a variety of eukaryotic cell lines. In humans and other mammals, these small RNAs help to sculpt the expression of most mRNAs (Bartel, 2018). Almost all brain miRNAs are co-expressed at different levels in different brain regions. There is also evidence that cell types (neurons and glial cells) specific miRNAs exist in the central nervous system, and they play a role in neuronal differentiation and synaptic plasticity.

In all, epigenetic modifiers, especially miRNAs, have received more and more scientific attention because of their role in stress sensitivity after early stress (Wigner et al., 2020). In addition to DNA methylation, other expression forms of epigenetic regulation (such as miRNA interference) may also play a key role in gene expression levels. These findings provide strong support for the argument that analysis of mRNA and protein expression levels and promoter methylation status can help understand the pathogenesis of mental illness (including depression) and the mechanism of action of drugs that effectively treat it.

CONCLUSION AND DISCUSSION

Early life stress has been known as a major cause for MDD, but how early life stress affects adult monoaminergic activity is not clear either. Recently, DNA methylation is considered to be the key mechanism of epigenetics and might play a role in early life stress induced mental illness. There are many studies supporting the hypothesis that DNA methylation of *MAO A* gene might the reason for depression. Consistently, decreased methylation of *MAO A*, which may reduce monoamine utilization by increasing *MAO A* activity, has been found in depressive patients, anxiety and affective disorders. This is consistent with the monoamine depletion hypothesis of anxiety and depression, which might be the mechanism of action of *MAO A* inhibitors (such as tranylcypromine, phenelzine, or moclobemide) that are successfully used to treat depression and social phobia (Shulman et al., 2013).

In the current review, we presented evidence of epigenetic changes in depression. The effects of different pressure sources on DNA methylation were sorted out. Looking ahead, it is important to carefully study the unique mechanism by which different types

of stressors alter DNA methylation. Here, we propose MAO DNA methylation as an important candidate mechanism for the onset of depression. Finally, it is very valuable to explore the differences in miRNA function due to pressure changes. Combining whole-genome expression and *in vitro* studies, these techniques can help us clarify the importance of DNA/RNA methylation in depression. It will help design a more personalized treatment plan for people with depression or suicidal behavior. As MDD involves a complexity of epigenetic regulation, and a large number of brain regions are closely related to stress-related psychopathology, studies trying to understand the interaction between ELS and epigenetics and adult neuropsychiatric diseases urgently need to improve specificity. In view of the above challenges, more advanced analytic models, including machine learning and bioinformatics, are urgently needed. Improving our understanding of epigenetic mechanisms involved in MDD can pave the way for the development of therapeutic and diagnostic interventions.

Even identical twins show substantial individual differences, which might be produced epigenetically by the two-way interaction between the brain and hormones, the immune system mediator and the autonomic nervous system (McEwen and Bulloch, 2019). Epigenetics shape the structure and function of the brain and other body systems, and the brain and body systems show considerable adaptive plasticity throughout development and adult life. As genomic research has shed lights on mechanism of depression, the etiology of mental illness is progressing from the psychodynamic origin proposed by Freud to an organic approach and epigenetic derivation.

AUTHOR CONTRIBUTIONS

QX, SG, and FW planned the project. QX and MJ wrote the first draft. MJ, FW, BY, and SG made major revisions to the logic of this article. All authors approved the final version of the manuscript for submission.

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Early Life Stress- and Drug-Induced Histone Modifications Within the Ventral Tegmental Area

*Ryan D. Shepard and Fereshteh S. Nugent**

Department of Pharmacology, Edward Hebert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, United States

Psychiatric illnesses are a major public health concern due to their prevalence and heterogeneity of symptom presentation resulting from a lack of efficacious treatments. Although dysregulated dopamine (DA) signaling has been observed in a myriad of psychiatric conditions, different pathophysiological mechanisms have been implicated which impede the development of adequate treatments that work across all patient populations. The ventral tegmental area (VTA), a major source of DA neurons in the brain reward pathway, has been shown to have altered activity that contributes to reward dysregulation in mental illnesses and drug addiction. It has now become better appreciated that epigenetic mechanisms contribute to VTA DA dysfunction, such as through histone modifications, which dynamically regulate transcription rates of critical genes important in synaptic plasticity underlying learning and memory. Here, we provide a focused review on differential histone modifications within the VTA observed in both humans and animal models, as well as their relevance to disease-based phenotypes, specifically focusing on epigenetic dysregulation of histones in the VTA associated with early life stress (ELS) and drugs of abuse. Locus- and cell-type-specific targeting of individual histone modifications at specific genes within the VTA presents novel therapeutic targets which can result in greater efficacy and better long-term health outcomes in susceptible individuals that are at increased risk for substance use and psychiatric disorders.

Keywords: ventral tegmental area, VTA, epigenetic, histone, acetylation, methylation, dopaminylation

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Fang Pan,
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Reviewed by:
Deena Marie Walker,
Oregon Health and Science University, United States
Qing-song Liu,
Medical College of Wisconsin, United States
Hitoshi Morikawa,
University of Texas at Austin, United States

***Correspondence:**
Fereshteh S. Nugent
fereshteh.nugent@usuhs.edu

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INTRODUCTION

Psychiatric disorders pose an extraordinary challenge to healthcare professionals due to their high prevalence and distribution globally. This places an extreme burden on healthcare systems from both an economic and resource standpoint due to a lack of sufficient understanding of the development and progression of various psychiatric disorders. While various degrees of genetic and phenotypic heterogeneity exist among patients, exposure to environmental risk factors contributes to individual variability through their effects on developmental organization of functional connections within discrete brain networks. This creates challenges in the development of therapeutics that are efficacious, long lasting, and generalizable across patient populations.

It has become more apparent that environmental interactions can impact both the development and function of the central nervous system (CNS). Although some psychiatric diseases have been

found to have an underlying genetic basis due to mutations in the coding of the DNA itself, there has been increasing interest in the role of disease-based alterations to the epigenome (Klengel and Binder, 2015). Coined by Waddington, "epigenetic" changes refer to genomic modifications that do not alter the coding of DNA within an organism, but rather influence chromatin architecture which regulates the rate of gene transcription (Berger et al., 2009; Deans and Maggert, 2015). There are various changes to the epigenome that can either enhance or repress transcriptional rates such as histone modifications (Kouzarides, 2007), DNA methylation (Greenberg and Bourc'his, 2019), and non-coding RNAs (Wei et al., 2017). This focused review centers on the role of histone modifications within the ventral tegmental area (VTA)—one of the major sources of dopamine (DA) neurons in the mesolimbic reward pathway; however, we acknowledge there is an extensive role for other epigenetic mechanisms such as DNA methylation and non-coding RNAs in psychiatric disorders (Kuehner et al., 2019). Ultimately, this review will provide a succinct and up-to-date summary of major findings on different histone modifications observed in the VTA specifically following exposure to ELS and drugs of abuse as environmental risks for psychiatric disorders (**Figure 1**).

VTA Dysfunction in Psychiatric Illnesses

The VTA is a heterogeneous structure consisting of DA (Zhang et al., 2010), GABAergic (Margolis et al., 2012), and glutamatergic neurons (Yamaguchi et al., 2007; Hnasko et al., 2012) with DA neurons representing the greatest percentage in total cellular composition (Morales and Margolis, 2017). The VTA has been traditionally studied within the context of reward- and motivated learning in that exposure to naturally rewarding stimuli results in DA release which encodes for reward prediction errors that reinforce reward-related behaviors (D'Ardenne et al., 2008; Schultz, 2010; Cohen et al., 2012; Steinberg et al., 2014; Keiflin and Janak, 2015). However, in juxtaposition, VTA DA neurons also regulate aversion and incentive salience (Tan et al., 2012; Lammel et al., 2014; Root et al., 2018). The VTA receives various excitatory inputs from structures such as the medial prefrontal cortex (mPFC), lateral dorsal tegmental area (LDTg), lateral hypothalamus (LH), bed nucleus of stria terminalis (BNST), and the lateral habenula (LHb) (Au-Young et al., 1999; Tzschentke and Schmidt, 2000; Georges and Aston-Jones, 2001; Omelchenko and Sesack, 2005; Caille et al., 2009; Lammel et al., 2012; Kempadoo et al., 2013; Brown and Shepard, 2016). Additionally, VTA DA neurons receive inhibitory signals from GABAergic neurons within the VTA (Margolis et al., 2012), as well as from the rostromedial tegmental nucleus (Kaufling et al., 2009) (RMTg; sometimes referred to as the "tail of the VTA") and nucleus accumbens (NAc) (Matsui et al., 2014; Edwards et al., 2017). Stimulation of these GABAergic populations restrain the release of DA from the VTA (Matsui and Williams, 2011; Poller et al., 2011; van Zessen et al., 2012; Matsui et al., 2014), but these GABAergic structures can also indirectly mediate the inhibitory effects on DA signaling from glutamatergic projections, such as the LHb (Ji and Shepard, 2007; Matsumoto and Hikosaka, 2007; Omelchenko et al., 2009; Brown et al., 2017). Of interest, VTA GABAergic interneurons also receive direct GABAergic inputs from the NAc which promote DA disinhibition (Xia et al., 2011) and therefore can contribute to reward dysregulation independent of DA signaling (Brown et al., 2012; Creed et al., 2014). VTA glutamatergic and GABAergic synapses exhibit both drug-induced and stress-induced plasticity (Nugent et al., 2007; Niehaus et al., 2010; Bellone and Luscher, 2012; Polter and Kauer, 2014; Authement et al., 2015; Langlois and Nugent, 2017; Polter et al., 2018), demonstrating how synaptic dysfunction at distinct, yet interconnected neural circuits, can dysregulate VTA DA neuronal responses and promote the formation of more habitual and compulsive stress-/drug-related behaviors. Importantly, stress and drugs of abuse "hijack" the brain reward circuitry and alter DA signaling from the VTA (Volkow and Morales, 2015; Langlois and Nugent, 2017; Bellone et al., 2020; Doyle and Mazei-Robison, 2020). One of the hallmarks of depression is anhedonia (inability to perceive pleasure) which can also result from aberrant VTA DA signaling that mediates reward deficits (Heshmati and Russo, 2015; Belujon and Grace, 2017). Human imaging studies have proven useful in helping to corroborate the role of DA dysregulation in psychiatric illnesses and substance use disorders (SUDs) (Volkow et al., 2007; Koob and Volkow, 2010; Shen et al., 2012). Altogether preclinical and clinical data highlight that VTA DA dysfunction is a major contributor in the pathophysiology of reward dysregulation and psychiatric disorders.

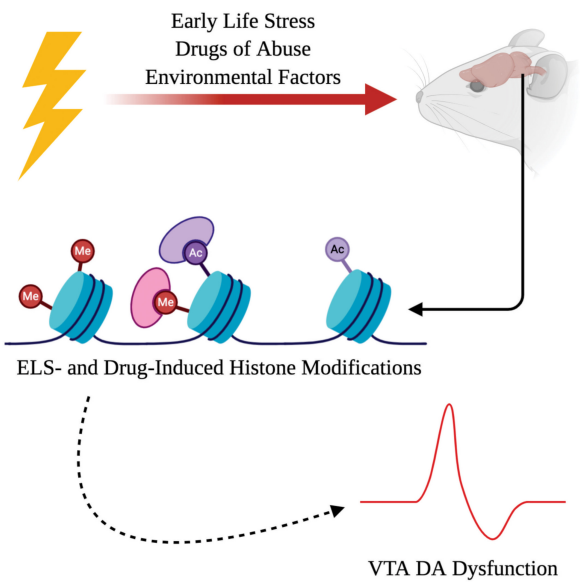

FIGURE 1 | Adverse interactions with the environment, such as early life stress (ELS) or exposure to drugs of abuse, can induce chromatin remodeling as a result of histone modifications. ELS- and drug-induced histone modifications can drive dopamine (DA) dysfunction within the ventral tegmental area (VTA) which contributes to the psychopathology observed in psychiatric illnesses.

Histone Modifications

Histone modifications dynamically regulate the chromatin structure that influences the rate at which genes are accessed

for transcription and subsequent translation. Transcriptional regulation has been widely demonstrated as an important set of processes that can both temporally and spatially define expression patterns of critical proteins and substrates which underlie important processes such as synaptic plasticity and neuronal excitability (Jiang et al., 2008; Sultan and Day, 2011). Specifically, alterations to chromatin structure are impacted by chromatin association with histones, which are octomeric proteins consisting of a combination of four different protein subunits: H2A, H2B, H3, and H4 (Luger et al., 1997); together, these form the nucleosome. These protein subunits have amino acid residues (also referred to as histone tails) that can be chemically modified by various enzymes (Allfrey and Mirsky, 1964; Allfrey et al., 1964). Chemical modifications to these amino acid residues affect how tightly associated DNA chromatin is bound to the histone. Thus, the chromatin state can either be more tightly associated or more relaxed with respect to the physical distance from the histone. A multitude of histone modifications have been documented including: methylation (Greer and Shi, 2012), acetylation (Graff and Tsai, 2013), phosphorylation (Rossetto et al., 2012), palmitylation (Wilson et al., 2011), polyADP-ribosylation (Martinez-Zamudio and Ha, 2012), sumoylation (Shiio and Eisenman, 2003), and ubiquitination (Zhang, 2003). Most recently, serotonylation (Farrelly et al., 2019; Fu and Zhang, 2019; Zlotorynski, 2019) and dopaminylation (Lepack et al., 2020) are two new types of histone modifications that have been observed. The importance of temporal regulation of the chromatin structure via histone modifications cannot be underestimated due to its importance in human development and the possible contribution of "histone code" alteration in disease (Borrelli et al., 2008).

Acetylation

Histone acetylation is the most understood histone modification in the CNS (Maze et al., 2013) and is dynamically regulated by two different classes of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs transfer acetyl groups to histone tails which results in chromatin relaxation, generally increasing transcription rates; HDACs remove acetyl groups which increase the chromatin-histone interaction and thus decrease the rate of transcription (Marmorstein and Zhou, 2014). The role of HATs in the VTA have not been extensively studied; however, one interesting study highlighted that cocaine abstinence promoted an increase in BDNF transcripts due to histone acetylation by CREB binding protein (CBP) in the VTA (Schmidt et al., 2012). Given this observation, further investigation into the contribution of HATs in psychiatric illnesses is warranted.

In contrast, HDACs have received the most attention due to their role in learning and memory (Graff et al., 2014; Mahgoub and Monteggia, 2014). Briefly, histone deacetylases can be classified based on the co-factor required for their activation, being either zinc-dependent or NAD-dependent (Haberland et al., 2009). The zinc-dependent HDACs consist of class I (HDAC1, 2, 3, and 8), class II (HDAC4, 5, 6, 7, 9, and 10), and class IV (HDAC11). NAD-dependent HDACs are also referred to as sirtuins (SIRT1, 2, 3, 4, 5, 6, 7, 8). Importantly, class I, II, and IV HDACs are primarily neuronal specific with class I HDACs residing in the nucleus and class II HDACs shuttling between nucleus and cytoplasm (Broide et al., 2007).

Several studies from our lab and other groups have corroborated the role of HDAC-mediated alterations to VTA histone acetylation that contributes to VTA DA dysfunction. Using a rat model of ELS (maternal deprivation, MD), we have found that MD induces GABAergic metaplasticity at GABAergic synapses onto VTA DA neurons that preferentially promotes long-term depression (LTD) (Authement et al., 2015). Moreover, MD-induced GABAergic synaptic dysfunction in VTA DA neurons was associated with altered A-kinase anchoring protein (AKAP150) signaling and decreased BDNF abundance possibly through HDAC2-mediated histone modification (Shepard et al., 2018). Specifically, HDAC2 was upregulated in VTA DA neurons with concomitant decreases in acetylation at H3K9 in protein isolates harvested from VTA tissues. Additionally, MD also increased VTA DA neuronal excitability involving altered AKAP150 signaling (Shepard et al., 2020). Interestingly, administration of CI-994 (a selective class I histone deacetylase inhibitor, HDACi) recovered GABAergic plasticity, histone acetylation, BDNF abundance, (Authement et al., 2015; Shepard et al., 2018), and VTA DA excitability (Shepard et al., 2020). Indeed, transcriptional regulation of the *Bdnf* gene through histone modifications including deacetylation of H3K9 via HDAC2 has also been reported (Chen et al., 2003; Wang et al., 2014; Chen and Chen, 2017). This raises the possibility that MD may lead to transcriptional repression of *Bdnf* gene in VTA DA neurons through increased HDAC2 occupancy at *Bdnf* promoters and histone H3K9 deacetylation in specific *Bdnf* gene promoters. MD-induced dysregulation of BDNF signaling in the VTA could potentially impact synaptic plasticity and VTA DA excitability through its effects on AKAP150 complex. In fact, BDNF signaling has previously been shown to regulate proteasome-dependent synapse remodeling and synaptic protein concentrations including the scaffold protein AKAP150 (Jia et al., 2008). Recent evidence also suggests that the reduction of AKAP150 from the postsynaptic density (PSD) via proteasomal degradation results in endocytosis of GluA1-containing AMPA receptors and promotion of LTD in mouse-cultured neurons (Cheng et al., 2020). Therefore, it will be worthwhile to investigate whether MD-induced epigenetic dysregulation of BDNF expression through HDAC2-histone hypoacetylation promotes degradation of AKAP150 at GABAergic synapses. This can act in concert with MD-induced disruption of AKAP150-PKA anchoring at GABAergic synapses (Shepard et al., 2018) to increase endocytosis of $GABA_A$ receptors and induce AKAP150-dependent LTD in VTA DA neurons (Dacher et al., 2013; Authement et al., 2015).

Drugs of abuse have also been shown to induce changes in histone acetylation (Renthal and Nestler, 2009), which possibly contribute to the etiology and maintenance of SUDs. Studies involving alcohol administration have also showed that alcohol increases HDAC2 expression, decreases acetylation of H3K9 and dysregulates $GABA_A$R signaling in the VTA; the effects of alcohol can be ameliorated with HDAC inhibition (Arora et al., 2013; You et al., 2018). In fact, it has been documented that the

metabolic breakdown of ethanol and production of acetate can contribute to changes in histone acetylation (Mews et al., 2019). Given that HDAC inhibition could attenuate alcohol-seeking behaviors, this further builds on evidence that dysregulated histone acetylation dynamics can contribute to maladaptive behaviors (Jeanblanc et al., 2015; Simon-O'Brien et al., 2015); however, whether these observations are exclusive to the VTA or involve other brain structures have not been fully elucidated. Morphine and its precursor, heroin, also have been observed to alter histone acetylation within the VTA (Authement et al., 2016; Xu et al., 2016). Moreover, acute exposure to morphine has been shown to dysregulate VTA synaptic transmission. Interestingly, HDACi treatment can normalize synaptic transmission and histone hypoacetylation in the VTA that are associated with acute morphine administration (Authement et al., 2016). Collectively, these studies highlight that HDAC-mediated histone modifications (such as histone hypoacetylation) within the VTA can contribute to transcriptional changes in BDNF signaling, AKAP150-dependent synaptic remodeling and stress- and drug-induced synaptic plasticity in VTA neurons that are reversible. Given that targeting HDACs with HDACi can ameliorate VTA synaptic dysfunction and the associated stress- and drug-related behaviors (Covington et al., 2009; Machado-Vieira et al., 2011), the use of HDACi could prove useful in treating or preventing neuropsychiatric disorders that stem from VTA dysfunction.

Methylation

Similar to histone acetylation, histone methylation is regulated by two classes of enzymes: histone methyltransferases (HMTs) and histone demethylases (HDMs). However, unlike histone acetylation, the addition and/or removal of methyl groups from histone tails by HMTs or HDMs, respectively, is not limited to a single chemical alteration. In fact, histone tails can either be mono-, di-, or tri-methylated depending on the amino acid residue which can have differential effects on the rate of transcription based on the methylation site (Black et al., 2012). Methylation of histones is accomplished by three families of HMTs that use S-adenosylmethionine (SAM) as the substrate that transfers the methyl group(s): (Su(var)3-9, enhancer of Zeste, trithorax)-domain containing proteins (Rea et al., 2000), DOT1-like proteins (Nguyen and Zhang, 2011), and protein arginine N-methyltransferases (PRMTs) (Di Lorenzo and Bedford, 2011). Demethylation of histones is carried out by two classes: amine oxidase LSD1 (KDM1) (Shi et al., 2004) and the Jumonji (JmjC) domain protein family (Klose et al., 2006; Tsukada et al., 2006). Given that histone acetylation can regulate VTA transcriptional activity and subsequently physiology, it is not surprising that alterations to histone methylation dynamics can also contribute to VTA pathophysiology.

In one study, decreased levels of BDNF transcript from VTA tissues were observed in samples obtained from both postmortem heroin users and rodent models (Koo et al., 2015). Chronic morphine was shown to increase trimethylation at both histone-3-lysine-4 (H3K4me3) and histone-3-lysine-27 (H3K27me3) which was associated with the BDNF II promoter. It was identified that H3K27me3 was mediated by the HMT enhancer of zeste homolog 2 (EZH2) in that overexpression and knockdown resulted in a decrease and increase of BDNF, respectively. Additionally, the actions of EZH2 on histone methylation impacted morphine's behavioral effects. However, only one study, the findings of this study, strongly suggests that overactivity of HMTs might be involved with drugs of abuse, such as morphine. Given that less is known about histone methylation, more research is required to understand if these alterations are specific to morphine or if they can extrapolate to other conditions.

Dopaminylation

Most recently, novel modifications to histones have been identified that can be modulated by neurotransmitters themselves: specifically, serotonylation (Farrelly et al., 2019; Fu and Zhang, 2019; Zlotorynski, 2019) and dopaminylation (Lepack et al., 2020). These intriguing findings suggest how dysregulated neurotransmission of monoamines, such as serotonin (5-HT) or DA themselves, can impact the epigenome to produce neuroadaptations involved in psychiatric diseases. Postmortem analysis of VTA tissue from cocaine users identified decreased dopaminylation of H3Q5 which was also observed in a rodent model of cocaine administration immediately after withdrawal; however, the levels of H3Q5 dopaminylation increased during prolonged withdrawal (30 days) (Lepack et al., 2020). This dopaminylation also was associated with altered transcription and increased release of DA into the NAc. Taken together, this novel finding opens up a previously unknown mechanism involved in histone regulation and suggests that monoamines themselves alters transcription of genes with subsequent changes in VTA physiology and related behaviors.

CONCLUSION

Precision-based medicine and advancements in genetic sequencing technologies have rapidly changed how we understand disease states, including psychiatric disorders. It has now become more greatly accepted that there is a genetic basis to many neuropsychiatric conditions. More importantly, the role of environmental modulation of epigenetic processes is being established through human and animal studies. This also poses an important and interesting question as to whether epigenetic alterations can be inherited (Heard and Martienssen, 2014). Transgenerational epigenetic inheritance has been documented in the case of DNA methylation (Sen et al., 2015), but not yet in the case of histone modifications. This observation begs the question as to whether epigenetic modifications need to be targeted during a critical period or can be possibly used pre-emptively to prevent adaptations that will result in psychiatric illnesses later in life. Thus, targeting enzymes mediating histone modifications should be further investigated and represent new pharmacological targets. In fact, certain compounds such as HDACi are already used in the treatment of cancer (West and Johnstone, 2014) and are being considered for use in psychiatric conditions (Covington et al., 2009; Machado-Vieira et al., 2011). One of the most interesting recent discoveries has been the possible contribution of serotonylation (Farrelly et al., 2019;

Fu and Zhang, 2019; Zlotorynski, 2019) and dopaminylation (Lepack et al., 2020) to histone modifications—two previously unknown epigenetic mechanisms. These two new mechanisms of histone modification alone add a new layer of complexity when studying epigenetic contributions to psychiatric disorders, but also give exciting and new targets for the development of therapies.

Moreover, one of the largest challenges in assessing epigenetic alterations, such as histone modifications, is understanding how they affect the CNS with region- and cell-type and locus-specificity. Previous experimental approaches of either transcriptional activation or suppression make it difficult to dissect contributions of epigenetic alterations to physiology and behavior due to artificial changes in gene expression that are not physiological (Yim et al., 2020). The advent of new epigenome-editing tools offer opportunities for precise locus-specific post-translational histone modifications including histone acetylation and histone methylation at specific genes in a single cell type/brain region (Hamilton et al., 2018b; Xu and Heller, 2019; Yim et al., 2020). The emerging and exciting field of neuroepigenetic editing tools allows for interrogation of epigenetic modifications in discreet brain regions and provides the opportunity to study causal relationships between gene transcriptional activity and the subsequent neural plasticity and behavior. For example, drug-induced activation of *Fosb* gene through increased histone acetylation (Levine et al., 2005) and decreased histone methylation (Maze et al., 2010) at its promoter has been observed, although the functional relevance of these correlated global histone post-translational modifications to drug-related behavior has been limited. To overcome this limitation, locus-specific targeting of the *Fosb* promoter with engineered transcription factors fused to zinc-finger proteins (ZFPs) facilitated bidirectional regulation of ΔFosB expression in mouse NAc neurons. Upregulation and downregulation was achieved using the transcriptional activator, p65, which promoted histone acetylation at H3K9/14 and the transcriptional repressor, G9a (promoting histone methylation at H3 lysine 9 dimethylation, H3K9me2) specifically at *Fosb*, respectively. Additionally, this bidirectional regulation of *Fosb* gene expression through these locus-specific histone manipulations was sufficient to induce opposing cocaine-related behaviors. Furthermore, they provided a direct causal link between H3K9me2-mediated reduction in *Fosb*/Δ*Fosb* expression in mouse NAc and the promotion of depression- and anxiety-like behaviors after social stress (Heller et al., 2014). Similarly, in another study, the same viral-epigenetic approach was employed to induce these complementary histone modifications at the cyclin-dependent kinase 5 (Cdk5) locus in mouse NAc and found that Cdk5-targeted H3K9/14 acetylation promoted Cdk5 gene expression and cocaine-induced locomotor behavior and resilience to social stress while Cdk5-targeted H3K9me2 induced Cdk5 gene repression and suppressed cocaine-related behaviors (Heller et al., 2016). Cre-dependent cell type-specific expression of *Fosb*-ZFPs in NAc D1- versus D2-medium spiny neurons (MSNs) allowed for cell-specific interrogations and provided compelling evidence for the opposite roles of *Fosb*-targeted histone acetylation and methylation in D1 and D2 MSNs in social defeat stress behavior (Hamilton et al., 2018a). This study also further highlights the necessity to consider differences in cell type-specific alterations to epigenetic modifications and their relationship with neurophysiology and behavior.

Given the robust regulatory role of HDAC-mediated histone modifications in VTA DA function, future epigenetic research should also employ these invaluable *in vivo* neuroepigenetic editing approaches for studying the causal relationships between an epigenetic modification at a single locus induced by stress or drugs of abuse within the specific neuronal populations of the VTA to its downstream functional outcomes at the transcriptional, cellular, circuit and behavioral levels. This locus- and cell type-specific targeted epigenome mapping and manipulation could help create more specific interventions that have more reliable, long-lasting, and efficacious treatment options for patients with psychiatric disease.

AUTHOR CONTRIBUTIONS

RS and FN wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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Involvement of Oxytocin Receptor/Erk/MAPK Signaling in the mPFC in Early Life Stress-Induced Autistic-Like Behaviors

Jinbao Wei[1,2,3], Le Ma[2], Peijun Ju[1], Beibei Yang[1], Yong-Xiang Wang[2] and Jinghong Chen[1]**

[1] Shanghai Key Laboratory of Psychotic Disorders, Shanghai Mental Health Center, School of Medicine, Shanghai Jiao Tong University, Shanghai, China, [2] King's Lab, School of Pharmacy, Shanghai Jiao Tong University, Shanghai, China, [3] Hubei Key Laboratory of Wudang Local Chinese Medicine Research, Institute of Wudang Traditional Chinese Medicine, Taihe Hospital, Hubei University of Medicine, Shiyan, China

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Fushun Wang,
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Daniel Vaiman,
Institut National de la Santé et de la Recherche Médicale (INSERM), France
Weiwen Wang,
Institute of Psychology (CAS), China

***Correspondence:**
Jinghong Chen
chenjh_008@hotmail.com
Yong-Xiang Wang
yxwang@sjtu.edu.cn

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The neonatal or infant period is a critical stage for the development of brain neuroplasticity. Early life stresses in the neonatal period, including neonatal maternal separation (NMS), have adverse effects on an increased risk of psychiatric disorders in juveniles and adults. However, the underlying molecular mechanisms are not largely understood. Here, we found that juvenile rats subjected to 4 h daily NMS during postnatal days 1 to 20 exhibited autistic-like behavioral deficits without impairments in learning and memory functions. Molecular mechanism studies showed that oxytocin receptor (OXTR) in the medial prefrontal cortex of NMS rats was evidently downregulated when compared with control pups, especially in neurons. Erk/MAPK signaling, the downstream coupling signaling of OTXR, was also inhibited in NMS juvenile rats. Treatment with oxytocin could relieve NMS-induced social deficit behaviors and activated phosphorylation of Erk/MAPK signaling. Furthermore, medication with the inhibitor of H3K4 demethylase alleviated the abnormal behaviors in NMS rats and increased the expression of OXTR in the medial prefrontal cortex, which showed an epigenetic mechanism underlying social deficits induced by NMS. Taken together, these findings identified a molecular mechanism by which disruptions of mother–infant interactions influenced later displays of typical social behaviors and suggested the potential for NMS-driven epigenetic tuning of OXTR expression.

Keywords: early life stress, neonatal maternal separation, oxytocin receptor, Erk/MAPK signaling, H3K4Me3

INTRODUCTION

Early life, including early childhood, neonatal, or even infant period, is a time of significant brain development and, therefore, a time when early social experiences influence the development of the central nervous system and expression of behaviors in subsequent adolescence and adulthood (Cushing and Kramer, 2005; Anda et al., 2006; Tian et al., 2018). Emerging pieces of evidence

Abbreviations: ASD, Autism spectrum disorder; ELS, early life stress; H3K4me3, tri-methylation of lysine 4 on histone H3; HDMs, histone demethylases; HMTs, methyltransferases; mPFC, medial prefrontal cortex; NMS, neonatal maternal separation; OT, oxytocin; OXTR, oxytocin receptor.

suggest that adversities during early life may contribute to a greater risk of developing mental disorders such as autism spectrum disorder (ASD), anxiety, and depression (Chapman et al., 2004; Carr et al., 2013; Flouri et al., 2020). Thus, early life stress (ELS) events are a cause or a predisposing factor for psychiatric diseases later in life.

Daily separation of newborns from their mothers for some hours during the neonatal period known as neonatal maternal separation (NMS), a model of ELS that leads to a disruption of maternal care, may have important implications for offspring neurodevelopment and disease risk (Carlyle et al., 2012; Loewy et al., 2019). As proved in animal studies, NMS led to anxiety-like behavior, accompanied by increased activity of hypothalamic–pituitary–adrenal (HPA) axis in rats (Daniels et al., 2004; Odeon and Acosta, 2019), and impaired localized *de novo* protein translation at synaptic connections in the rat hippocampus (Ahmad et al., 2018). Moreover, ELSs may be involved in deficits in social recognition (Kambali et al., 2019). Our behavioral results indicated that maternally separated rats during adolescence showed impaired social novelty preference for strange rats and increased preference for marble-burying behavior. These impairments were typical performances of autistic-like behavioral deficits that were characterized by the core symptoms of impaired social recognition, language communication failure, and stereotyped behaviors (Ferguson et al., 2001). Recent studies have identified that oxytocin (OT) and the oxytocin receptor (OXTR) exert important roles in the regulation of complex social behaviors such as maternal behavior, mating, aggression, attachment, sexual behavior, as well as in psychiatric disorders characterized by social deficits (Ferguson et al., 2001; Bakermans-Kranenburg and van Ijzendoorn, 2014; Hoge et al., 2019). The neuropeptide OT produced by the supraoptic nucleus and paraventricular nucleus of the hypothalamus in the brain, and various peripheral organs and membranes (kidney, amnion, heart, uterus, etc.), has been revealed as a profound anxiolytic and anti-stress factor of the brain with activation Raf/MEK/Erk/CREB (Erk/MAPK) signaling, an extracellular signal-regulated kinase pathway, besides its reproductive and prosocial effects (Zhong et al., 2003; Aoki et al., 2014; Neumann and Slattery, 2016). Due to its functions on the improvement of social recognition, intranasal OT has been under clinical studies for use in the treatment of adults and juveniles with ASD (Anagnostou et al., 2012; Penagarikano, 2015). Furthermore, OXTR variants were involved in children subjected to emotional neglect (Womersley et al., 2019). Deficits by knockout of OXTR or OT could lead to pervasive social deficits in mice (Takayanagi et al., 2005). These findings suggest that OT/OXTR system is linked to psychiatric disorders associated with social deficits and may be a potential therapeutic target for social deficits in ASD. Early life adversity events exert long-lasting influences on the brain via epigenetic regulatory mechanisms, making an individual susceptible to later psychiatric disorders (Unternaehrer et al., 2015; Seo et al., 2016). Early life experiences could cause epigenetic variability of OXTR in the brain implicated in social behavior (Kraaijenvanger et al., 2019; Tops et al., 2019).

We found that OXTR in juvenile NMS rats was significantly downregulated than control littermate pups, accompanied by phosphorylation inhibition of its downstream Erk/MAPK signaling in the medial prefrontal cortex (mPFC), an important brain region that plays an essential role in mediating social cognition (Amodio and Frith, 2006). Due to the critical role of the OT/OXTR system in the regulation of social behaviors, we hypothesized that repressed OXTR/Erk/MAPK signaling was implicated in NMS-induced social deficit. Our results showed that OT treatment relieved the social deficit of NMS rats with activation of Erk/MAPK signaling, demonstrating that the OT signaling was involved in social deficit in NMS rats. OXTR expression was regulated via tri-methylation of lysine 4 on histone H3 (H3K4me3), a marker for transcriptional activation that could provide support for the increase in OXTR messenger RNA (mRNA) level (Greer and Shi, 2012; Miller, 2016). H3K4me3 level was regulated by histone demethylases (HDMs) and methyltransferases (HMTs), which were widely expressed in different functional brain regions including PFC, hippocampus, hypothalamus, etc. (Kouzarides, 2007). KDM5B, a critical HDM, served as the main regulatory factor of H3K4me3; knockdown of which by small interfering RNA could increase OXTR expression via upregulating H3K4me3 (Miller, 2016). It was found that NMS caused a decrease of H3K4me3 level in the mPFC. Consequently, our studies further investigated whether NMS stress changed OXTR expression through epigenetic modulation of the histone methylation pathway. These findings demonstrated that NMS-induced epigenetic changes at OXTR might be predictive adaptive outcomes that facilitate behavioral pathology.

MATERIALS AND METHODS

Animals

Timed-pregnant (15 days pregnant) Sprague-Dawley female rats were purchased from Shanghai Leigen Biotechnology Co., Ltd. All dams were housed in standard laboratory housing rooms with controlled temperature (23 ± 2°C) and humidity (25–70%) under a 12/12-h light/dark cycle (lights on at 7:30 a.m.). They were given standard food and drinking water *ad libitum*. Pregnant females were individually housed until delivery. In total, 94 male offsprings (assigned into four batches assays) were used in this study. All of the animal experiments were performed according to international guidelines and approved by the Experimental Animal Committee of Shanghai Jiao Tong University School of Medicine.

Neonatal Maternal Separation Procedure

The birth date was considered as postnatal day 0 (PND 0). When pups were born, they were subjected to the NMS paradigm at PND 1. The NMS paradigm, similar to previous protocols (Amini-Khoei et al., 2015; Amiri et al., 2016), was used with slight modifications. The male offsprings were separated from their mothers for 4 h daily during PND 9–15 for a short-term separation or PND 1–20 for a long-term separation, beginning at 10:00 a.m., and were returned to their mothers after the 4-h separation period. During the separation period,

the NMS offsprings were placed in separate compartments, and no nutritional supplements were provided. The offsprings were weaned on PND 21 and housed in groups (four rats per cage) until experiment day PND 38. The control littermates were undisturbed, weaned on PND 21, and grouped in cages ($n = 4$) till PND 38 for behavioral tests. All animals were handled for several times before the behavioral tests and acclimated to the behavior testing room 1 h to reduce stress and anxiety.

Drug Administration

This study of the pharmacological intervention was carried out in two different parts. For the first part, to investigate the effects of OT administration on NMS juvenile rats, OT (Sandoz, United Kingdom) was dissolved in sterile 0.9% saline and intraperitoneally (i.p.) (1 mg/kg, 30 min before every behavioral test, i.p.) administered to the animals (NMS + OT group, nine males). The control littermates (CONT group, nine males) and other NMS juvenile rats (NMS group, nine males) were treated with an equal volume of 0.9% saline (30 min before every behavioral test, i.p.).

For the second part, the HDMs inhibitor As-8351 (Sigma131 Aldrich, Germany) was dissolved in 100% dimethyl sulfoxide and diluted to a 25% dimethyl sulfoxide solution (vehicle) with 0.9% saline for i.p. injection of 2 mg/kg. As-8351 was daily administered to the animals (NMS + AS-8351 group, nine males) on PND 21–35. Correspondingly, the CONT group (nine males) and NMS group rats (nine males) were received with an equal volume of the vehicle on PND 21–35.

Behavioral Testing

Three-Chamber Sociability and Social Novelty

The three-chamber test is used to assess cognition in the form of general sociability and interest in social novelty in rodent models of psychiatric disorders (Qin et al., 2018). Thus, we used the three-chamber test to identify the effects of NMS on deficits in sociability and/or social novelty in juvenile female rats. Briefly, an apparatus (L: 60 cm, W: 40 cm, H: 22 cm, equipped with EthoVision XT 10 software package, Noldus Information Technology; Leesburg, VA, United States) containing three chambers with openings between the chambers allowing rats to access into side chambers was used. During the habituation, two empty capsules (an inverted pencil cup, D: 8 cm, H: 15 cm, placed in the center area) were placed at side chambers, and the subject was allowed to explore for 10 min. After habituation to the apparatus with two empty capsules, the subject was returned to the home cage. The durations staying in the side chambers were recorded. Those spending longer than 50 s in one side chamber than the other side were excluded in the following tests. For the sociability test, then, a never-before-met social rat (age- and sex-matched rat of the same strain, stranger rat 1, S1) was placed under one of the empty capsules, and the subject was placed in the center chamber and free to investigate the apparatus for 10 min. After the sociability measurement, the test subject was returned to the home cage. It took a 5-min interval for the next measurement. For the social novelty test, a new never-before-met social rat (stranger rat 2, S2) was placed under the other capsule, and the previous social rat now served as the familiar rat (S1). The subject then was placed again in the center chamber and free to investigate the apparatus for 10 min with a choice between the first, now-familiar, social rat (stranger 1) in one side chamber and a second unfamiliar rat (stranger 2) in the other side chamber. During the sociability and social novelty tests, the time spent sniffing each capsule, the time spent in each chamber, and the numbers of entries into each chamber were recorded and analyzed. After the tests, the two social rats and subjects were returned to their home cages. The chambers and capsules were cleaned with 75% ethanol after the tests.

Social Approach

As a more sensitive measure for sociability, we also used the social approach test to investigate the variation of social behavior in juvenile rats. After, the subject was placed in an apparatus (L: 58.5 cm, W: 58.5 cm, H: 58.5 cm) containing an empty capsule (an inverted pencil cup, D: 8 cm, H: 15 cm, placed in the center area) to explore for 10 min, then was returned to the home cage (Qin et al., 2018; Sharon et al., 2019). A never-before-met social rat (age- and sex-matched rat) was placed under the capsule. Subsequently, the subject was placed back into the apparatus and allowed to interact with the stimulus rat for 10 min, while the interaction time was recorded using an overhead video camera and tracked using the EthoVision XT 10 software package (Noldus Information Technology; Leesburg, VA, United States).

Novel Object Recognition

Rats show a preference to interact with a novel than with a familiar object. Novel object recognition test has been used to study learning and memory for behavioral and psychiatric disorders (Bevins and Besheer, 2006). To an apparatus (L: 58.5 cm, W: 58.5 cm, and H: 58.5 cm), two identical to-be-familiarized objects (cylindrical, D: 8 cm, H: 10 cm) were placed at the back left and right corners. The subject was placed at the midpoint of the wall opposite the objects in the apparatus, allowed to explore for 10 min, and returned to the home cage. After 1 h, one of the objects was replaced with a new object (cube, L: 10 cm, W: 10 cm, and H: 10 cm); the subject was placed back into the apparatus, being allowed to explore the two different objects for 10 min. The interaction time of exploring the two objects was recorded using an overhead video camera and analyzed using the EthoVision XT 8.5 software package (Noldus Information Technology; Leesburg, VA, United States).

Marble Burying

The marble-burying test is used for assessing the stereotyped and repetitive behavior relevant to ASD (Sharon et al., 2019). Marble burying was performed in a normal cage (L: 48 cm, W: 35 cm, and H: 20 cm) bottom with a floor area filled with 7–8 cm of fresh, autoclaved wood chip bedding (Sharon et al., 2019). The subject was habituated to the cage for 10 min, then returned to the home cage. Subsequently, the bedding was leveled, and 20 glass marbles (4×5) were placed on the top of the bedding. The subject was placed back into the cage and allowed to explore for 10 min. The number of buried marbles (50% or more covered) was recorded. The bedding was renewed for each subject, and marbles were cleaned with 75% ethanol.

Tissue Collection, RNA, and Protein Extraction

On PND 42, juvenile rats from different groups were accepted anesthesia by first administering i.p. 10% chloral hydrate and then decapitated. Subsequently, brains were split using surgical scissors, and the brain region of Mpfc was collected into an RNase-free tube and kept frozen at −80°C until analysis. To investigate the effects of OT or AS-8351 administration in NMS rats, the brain tissue was collected after the final behavioral test of administration within 1 h on PND 42. Total RNA was extracted from mPFC tissue using RNA extraction reagent RNAiso Plus (Code No. 9109, TaKaRa BIO INC., Japan). Protein was extracted from PFC by radioimmunoprecipitation assay lysis buffer (Beyotime Biotechnology, China) with 1-mM phenylmethylsulfonyl fluoride and cocktail protease inhibitor (Roche, United States), boiled in 4× sodium dodecyl sulfate loading buffer for 5 min and then kept frozen in −20°C after being cooled to room temperature.

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time PCR was used to compare the mRNA levels. PrimeScript^{TM} RT reagent Kit with gDNA Eraser (TaKaRa BIO INC., Japan) was used to synthesize complementary DNA. Real-time PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech, United States) and analyzed using LightCycler® 480 SYBR Green Software II (Roche, United States). The executive programs of amplification were the following parameters: 95°C for 30 s, followed by 45 cycles of heating at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 95°C for 15 s. Sequences of the primers were as follows: OXTR, forward ACCTGGATATGCGCAAGTGT, reverse GGGCAGGTAGTTCTCCTCCT; glyceraldehyde phosphate dehydrogenase (GAPDH), forward GACATGCCG CCTGGAGAAAC, reverse AGCCCAGGATGCCCTTTAGT. The relative expression of the respective genes was normalized to that of GAPDH within the same sample. ΔCt, indicating the difference between GAPDH and the target gene, was expressed by the formula $\Delta Ct = Ct_{targetgene} - Ct_{GAPDH}$, ΔΔCT was expressed by the formula ΔΔCT = ΔCT (treated group) − ΔCT (CONT group), and the relative expression of each mRNA level was calculated using the $2-^{\Delta\Delta Ct}$ method. The final value was represented as a value relative to the control group.

Western Blotting

The protein samples from mPFC were separated on 12–8% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride filter (0.45 μm, Millipore, United States). The membrane was blocked by non-fat milk (5%) in Tris-buffered saline containing 0.1% Tween 20 and incubated with primary antibodies against including GAPDH (1:1,000, Affinity Biosciences, United States), OXTR (1:800, Abcam, United Kingdom), tri-methyl-histone H3 Lys4 (1:1,000, Cell Signaling Technology, United Kingdom), *p*-Erk1/2 (1:1,000, Cell Signaling Technology, United States), Erk1/2 (1:1,000, Cell Signaling Technology, United States), *p*-MEK1/2 (1:1,000, Cell Signaling Technology, United States), MEk1/2 (1:1,000, Cell Signaling Technology, United States), *p*-c-Raf (1:1,000, Cell Signaling Technology, United States), c-Raf (1:1,000, Cell Signaling Technology, United States), H3 (1:1,000, Cell Signaling Technology, United States), CREB (1:800, Cell Signaling Technology, United States), and *p*-CREB (1:800, Cell Signaling Technology, United States) overnight at 4°C. After 1-h incubation at 37°C with secondary antibodies including horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Affinity Biosciences, United States), HRP-conjugated goat anti-rabbit IgG (1:10,000, Absin, China), and HRP-conjugated goat anti-mouse IgG (Affinity Biosciences, United States) followed by visualization under iBright FL1000 imaging system (Thermo Fisher, United States) with chemiluminescence (ECL Advance; Amersham Biosciences). Protein band intensity was quantified using Image J software (National Institutes of Health, Bethesda, MD, United States), and the relative expression level of respective proteins was calculated by normalization to the GAPDH protein.

Immunohistochemistry

Immunofluorescence staining was performed after behavioral tests. Rats were anesthetized with 10% chloral hydrate, followed by perfusion with 0.9% saline and 4% paraformaldehyde solutions. Brains were postfixed overnight in 4% paraformaldehyde at 4°C followed by dehydration with gradient sucrose in phosphate-buffered saline. Rat brain sections (30 μm) were blocked with 3% bovine serum albumin in 0.3% Triton X-100 for 1 h at room temperature and incubated for 24 h at 4°C with primary antibodies including OXTR (1:600, Abcam, United Kingdom), IBA-1 (1:500, Wako, Japan), NeuN (1:600, Cell Signaling Technology, United States), and glial fibrillary acidic protein (GFAP) (1:800, Proteintech, United States). After washing with phosphate-buffered saline three times for 30 min, the sections were incubated for 2 h at room temperature with secondary antibodies Alex Flour® 594-conjugated goat anti-rabbit (1:600, Abcam, United Kingdom) and Alex Flour® 488-conjugated donkey anti-goat (1:600, Absin, China). Expressions of OXTR, IBA-1, GFAP, and NeuN in mPFC were visualized under a laser scanning confocal microscope (Olympus, Japan). Nucleic staining reagent 4′,6-diamidino-2-phenylindole fluoromount-G (Southern Biotech, United States) was used to stain cell nuclei. Colocalization analysis was identified via utilizing ImageJ software (National Institutes of Health, Bethesda, MD, United States) that was equipped with a colocalization finder, under which colocalized pixels appeared white. Merged confocal images (20×) of neural cell markers (IBA-1, NeuN, GFAP, red) costained with OXTR (green) and 4′,6-diamidino-2-phenylindole (blue) in mPFC slices of CONT and long-term NMS rats were shown.

Statistics

Data analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, United States). For

statistical significance, unpaired, two-tailed Student's *t*-test was used to evaluate statistical significance between the two groups. One-way with Tukey's *post hoc* tests or two-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni tests were used to evaluate statistical significance in experiments with more than two groups. Two-way repeated measure ANOVA followed by *post hoc* Bonferroni tests was used to evaluate the weight loss of NMS. The level of statistical significance was set at $P < 0.05$. *F* values, degrees of freedom, and *P* values for all ANOVAs (representing statistics of *post hoc* comparisons and main interaction effects, unless otherwise stated), as well as degrees of freedom for *t*-tests and *t*-values, are shown in figures. Data were presented as mean ± SEM.

RESULTS

Short-Term Neonatal Maternal Separation Stress Did Not Influence Social Recognition in Juvenile Rats

Early life stress confers lifelong stress susceptibility (Pena et al., 2017) and causes psychiatric diseases and cognitive impairment later in life, including social deficits relevant to ASD, anxiety, and depression (Chapman et al., 2004; Carr et al., 2013; Flouri et al., 2020). To examine the effects of ELS during the stress hyporesponsive period on social and cognitive–behavioral changes in juvenile rats, we used NMS as the ELS. The NMS offsprings were subjected to a daily 4 h maternal separation on PND 9–15 (**Figure 1A**), whereas the control littermates were kept with their mother without disturbances. After a short-term (a week) NMS stress in neonatal rats, behavioral tests were conducted on PND 36–42.

Here, the three-chamber test was used to assess cognitive changes induced by NMS in the form of general sociability and interest in social novelty, which was useful for quantifying deficits in social behavior in animals exhibiting autistic traits (Qin et al., 2018). Our behavioral results indicated that both the juvenile controls and the rats suffering from short-term NMS showed a similar preference for spending more time in the chamber containing a stimulus rat compared with the empty chamber (**Figure 1B**, two-way ANOVA followed by the *post hoc* Bonferroni's tests, n.s. [not significant], $F_{(1,36)} = 1.975$, $P = 0.1685$; $p > 0.05$). We also used the social approach test to investigate the effect of short-term NMS on the sociability of juvenile rats. It showed that NMS rats spent similar time investigating the social rat (**Figure 1D**, two-tailed unpaired *t*-test, $t_{18} = 0.4486$, $P = 0.6591$; n.s., $p > 0.05$), suggesting that NMS rats did not show impairment in sociability. Likewise, no differences in social novelty were observed between the controls and NMS rats (**Figure 1C**, two-way ANOVA followed by the *post hoc* Bonferroni's tests, $F_{(1,36)} = 1.821$, $P = 0.1425$; n.s., $p > 0.05$), which demonstrated that social novelty was not impaired in NMS rats. Furthermore, in the marble-burying test, NMS rats did not show repetitive and stereotypical behavior compared with the controls (**Figure 1E**, two-tailed unpaired *t*-test, n.s., $t_{18} = 0.596$, $P = 0.5973$; $p > 0.05$). These data discussed suggested that exposition to short-term NMS did not trigger social deficits and induced ASD-like behaviors in rats during juvenile.

Long-Term Neonatal Maternal Separation Stress Showed Impacts on Social Recognition but Not Learning and Memory Functions

As shown in **Figure 1**, short-term NMS did not affect the social cognition, including sociability and social novelty, and cause autistic-like behaviors; we thought that the different durations of stress in rodents might bring different behavioral outcomes. A previous study has revealed that the rats daily suffering from a maternal separation for 80 min on PND 2–14 showed more increased anxiety-like behaviors, susceptibilities to stress, and locomotor activities compared with the rats for 15 min on PND 2–14 (Lippmann et al., 2007). Hence, we further explored the effects of long-term NMS stress on cognitive functions in juvenile rats. For the long-term NMS procedure, the NMS offsprings were subjected to a daily 4-h maternal separation on PND 1–20 and then weaned on PND 21 (**Figure 2A**).

It is well known that weight loss is an independent risk and biomarker for mental illness and developmental impairment. During NMS, normal maternal lactation was interrupted. As a result, the bodyweight of long-term NMS rats was significantly lower than that of the control rats during PND 21–42 (**Figure 2B**, two-way repeated-measures ANOVA followed by the *post hoc* Bonferroni's tests, $F_{(3,72)} = 5.382$, $P < 0.0001$). The behavioral results showed the rats experiencing long-term NMS still showed a similar preference for spending more time in the chamber containing a stimulus rat compared with the empty chamber (**Figure 2C**, two-way ANOVA followed by the *post hoc* Bonferroni's tests, $F_{(1,36)} = 2.013$, $P = 0.1645$; n.s.), and the social approach test observed no differences in sociability between them (**Figure 2E**, two-tailed unpaired *t*-test, $t_{18} = 1.032$, $P = 0.3155$; n.s.), suggesting that long-term NMS did not have serious consequences on sociability. In the social novelty test, NMS rats spent less time in exploring the chamber containing a social rat in comparison with the controls, which suggested that NMS rats exhibited a lower preference for the stranger rat (**Figure 2D**, two-way ANOVA followed by the *post hoc* Bonferroni's tests, $F_{(1,36)} = 32.32$, $P < 0.0001$). It has been reported that a lack of preference for social novelty is implicated in impairments in social recognition or memory, which are important early markers for ASD and neurodevelopmental illnesses with strong genetic susceptibility (Frazier and Goodwin, 2020). To further assess whether long-term NMS could cause repetitive and stereotypical behavior, a behavioral deficit involved in ASD, we performed the marble-burying test. The results showed that the juvenile rats subjected to long-term NMS exhibited repetitive and stereotypical autistic-like behavior compared with the control littermates (**Figure 2F**, two-tailed unpaired *t*-test, $t_{18} = 2.634$, $P = 0.0168$). In addition, in consideration that long-term NMS could lead to impairments in social recognition, we used novel object recognition to further investigate whether it affected the learning and recognition memory functions.

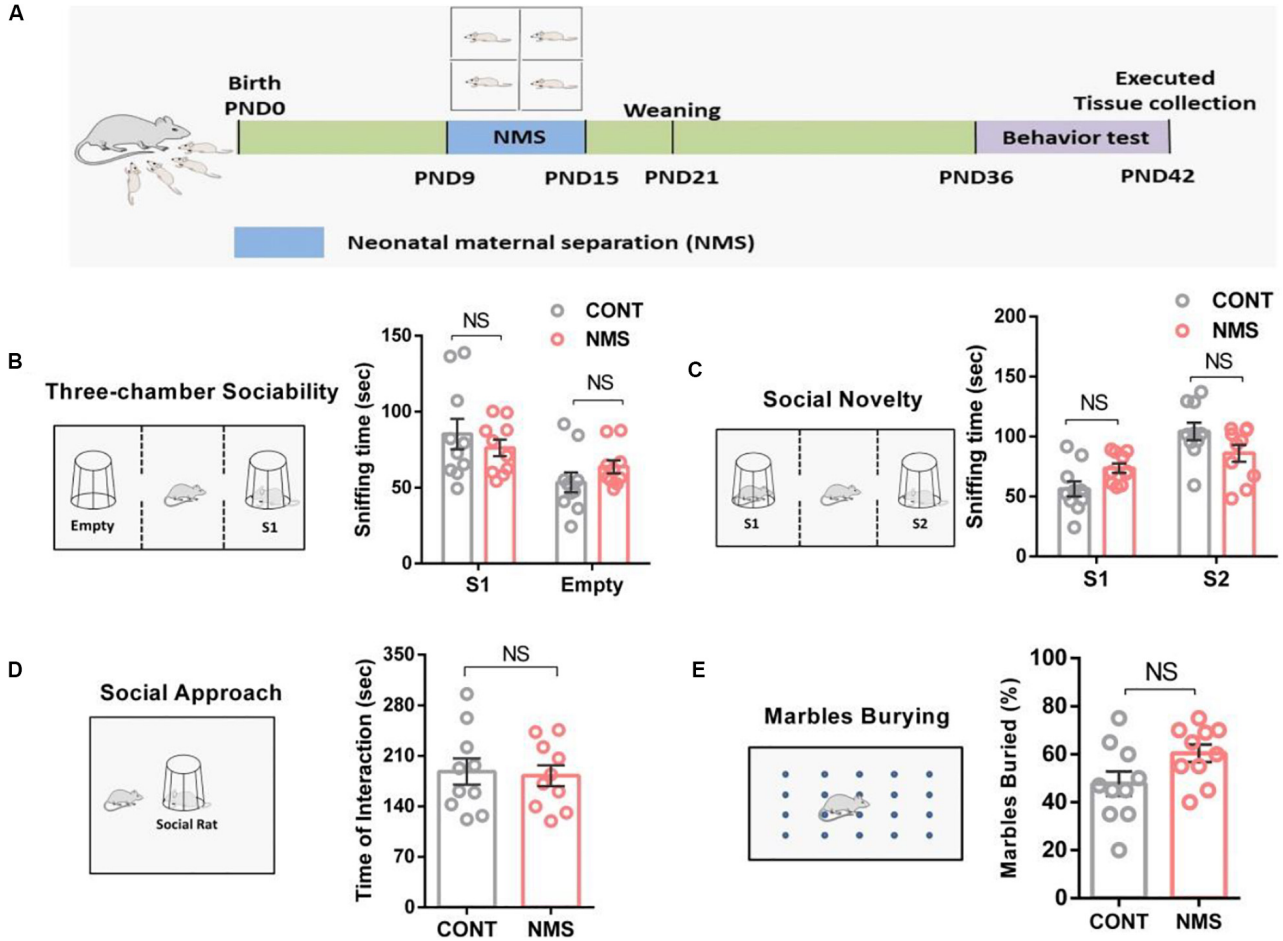

FIGURE 1 | Juvenile rats suffering from short-term neonatal maternal separation did not display behavioral social deficits. **(A)** Experimental design: neonatal rats were subject to a short-term maternal separation from PND 9–15. **(B)** Three-chamber sociability test, plots showing the time spent investigating either the social (S1) or non-social (Empty) stimulus (two-way ANOVA followed by the *post hoc* Bonferroni's multiple comparison tests, $F_{(1,36)} = 1.975$, $P = 0.1685$; n.s., $P > 0.05$). **(C)** Social novelty test, plots showing the time spent investigating either the familiar (S1) or unfamiliar (S2) social rat (two-way ANOVA followed by the *post hoc* Bonferroni's multiple comparison tests, $F_{(1,36)} = 1.821$, $P = 0.1425$; n.s., $P > 0.05$). **(D)** Social approach test, plots showing the time spent investigating the social rat (two-tailed unpaired *t*-test, $t_{18} = 0.4486$, $P = 0.6591$, n.s., $P > 0.05$). **(E)** Marble-burying test, plots showing the percentage of buried marbles (two-tailed unpaired *t*-test, $t_{18} = 0.596$, $P = 0.5973$, n.s., $P > 0.05$). n.s., not significant. Data are represented as mean ± SEM ($n = 10$ in each group).

As shown in the novel object recognition test, both the NMS rats and the controls displayed a similar preference for spending more time in the novel object than the familiar object (**Figure 2G**, two-way ANOVA followed by the *post hoc* Bonferroni's tests, $F_{(1,36)} = 1.543$, $P = 0.2222$; n.s.), demonstrating that long-term NMS did not trigger deficits in learning and memory functions.

Long-Term Neonatal Maternal Separation Induced Oxytocin Receptor Downregulation and Inhibition of Erk/MAPK Signaling in Medial Prefrontal Cortex

Experiences in early life have shown the potential to change social recognition and may lead to neuropsychiatric disorders, including ASD (Tabbaa et al., 2017; Brydges et al., 2019). As we found earlier, long-term repetitive NMS could cause impairments in social recognition and autistic-like behaviors. However, the underlying molecular mechanisms have still not been well understood. It has been found that early life experiences may influence behaviors via epigenetic-mediated mechanisms (Babenko et al., 2015). Recent studies have revealed that early rearing history affects OXTR epigenetic regulation in rhesus macaques and prairie voles, which may be involved in behavioral deficits later in life (Baker et al., 2017; Perkeybile et al., 2019).

Based on these recent findings, we hypothesized that OXTR might be implicated in long-term repetitive NMS-induced impairments in social recognition and autistic-like behaviors. Our data showed that OXTR gene expression in NMS juvenile rats was markedly downregulated in mPFC (**Figure 3A**, two-tailed unpaired *t*-test, $t_4 = 7.869$, $P = 0.0014$),

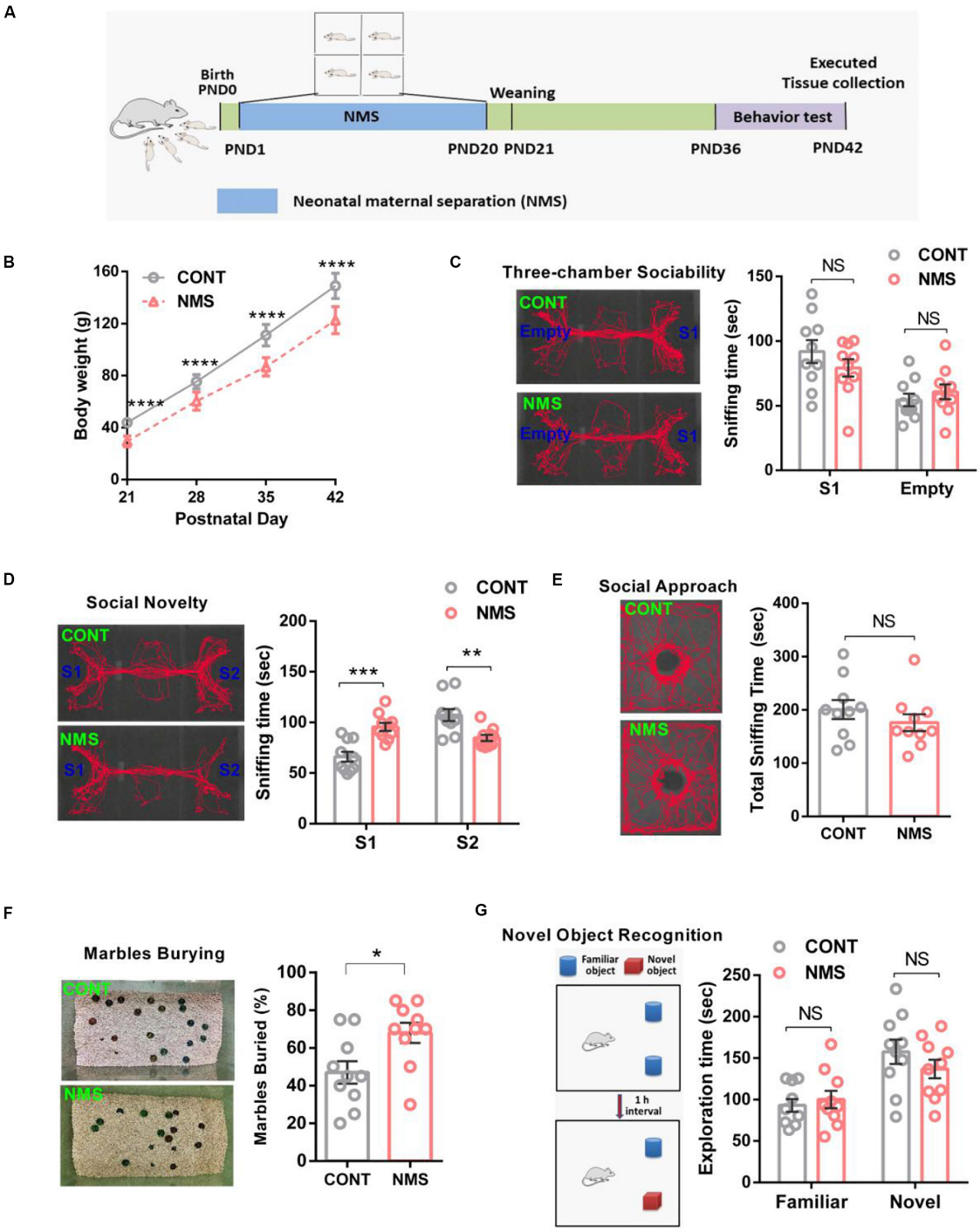

FIGURE 2 | Effects of long-term neonatal maternal separation stress on bodyweight, social recognition, and memory recognition in juvenile rats. **(A)** Experimental design: neonatal rats were subject to a long-term maternal separation from PND 1–20. **(B)** Bodyweight at different time points (two-way repeated-measures ANOVA followed by the *post hoc* Bonferroni's multiple comparison tests; $F_{(3,72)} = 5.382$, $P < 0.0001$, CONT vs NMS, $^{****}P < 0.0001$). **(C)** Three-chamber sociability test, plots showing the time spent investigating either the social (S1) or non-social (Empty) stimulus (two-way ANOVA followed by the *post hoc* Bonferroni's multiple comparison tests; $F_{(1,36)} = 2.013$, $P = 0.1645$, NS, $P > 0.05$). **(D)** Social novelty test, plots showing the time spent investigating either the familiar (S1) or unfamiliar (S2) social rat (two-way ANOVA followed by the *post hoc* Bonferroni's multiple comparison tests; $F_{(1,36)} = 32.32$, $P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$). **(E)** Social approach test, plots showing the time spent investigating the social rat (two-tailed unpaired *t*-test, $t_{18} = 1.032$, $P = 0.3155$, n.s., $P > 0.05$). **(F)** Marble-burying test, plots showing the percentages of buried marbles (two-tailed unpaired *t*-test, $t_{18} = 2.634$, $P = 0.0168$, $^{*}P < 0.05$). **(G)** Novel object recognition test, plots showing the time spent investigating either the familiar or novel object (two-way ANOVA followed by the *post hoc* Bonferroni's multiple comparison tests; $F_{(1,36)} = 1.543$, $P = 0.2222$, n.s., $P > 0.05$); n.s., not significant. Data are represented as mean ± SEM ($n = 10$ in each group).

an important brain region that played an essential role in modulating social cognition, when compared with the controls littermates. The OXTR mRNA expression in mPFC was also dramatically decreased in NMS rats (**Figure 3B**, two-tailed unpaired *t*-test, $t_4 = 4.422$, $P = 0.0115$). Consequently, our results indicated that long-term repetitive NMS could downregulate the protein and mRNA levels of OXTR in the mPFC of NMS rats.

OT, as the endogenous ligand of OXTR, exerting effects via OXTR, has been shown to improve social symptoms in ASD patients by intranasal administration due to its roles in regulating social behavior and improving anxiety (Yoshida et al., 2009; Yatawara et al., 2016; Parker et al., 2017). Rodents bearing knockout for either the OT gene or OXTR gene showed conspicuous social deficits (Modi and Young, 2012). As the expression of OXTR, in our findings, was downregulated in NMS rats, we then further investigated whether the downstream signaling of OXTR, namely, c-Raf/MEK/Erk/CREB pathway, was affected. Our results indicated that the phosphorylation of c-Raf/MEK/Erk/CREB signaling in mPFC was dramatically inhibited (**Figures 3C–G**, two-tailed unpaired *t*-test; $t_4 = 4.958$ [*p*-c-Raf], $P = 0.0077$; $t_4 = 4.687$ [*p*-MEK1/2], $P = 0.0094$; $t_4 = 25.46$ [*p*-Erk1/2], $P < 0.0001$; $t_4 = 3.200$ [*p*-CREB], $P < 0.0329$). Thus, the data suggested that long-term repetitive NMS could induce OXTR downregulation with inhibition of the downstream Erk/MAPK signaling.

Oxytocin Treatment Improved Social Deficient Behavior in Juvenile Long-Term Neonatal Maternal Separation Rats Accompanied With Activation of Erk/MAPK Signaling

Intranasal administration of OT is clinically used in patients with autism for improving social deficits (Modi and Young, 2012; Yatawara et al., 2016). Herein, we used OT to treat the juvenile rats subjected to NMS to examine its effects on NMS-induced autistic-like behaviors. It showed that OT administration was shown to decrease repetitive and stereotyped behavior in the marble-burying test (**Figure 4A**, one-way ANOVA followed by the *post hoc* Tukey's tests, $F_{(2,24)Treatment} = 4.983$, $P = 0.0155$) and rescue the impairments in social recognition by increasing the preference for the strange rat in social novelty test (**Figure 4B**, one-way ANOVA followed by the *post hoc* Tukey's tests, $F_{(2,46)} = 20.50$, $P = 0.0003$), indicating that OT treatment could improve NMS-induced social deficient and ASD-like behaviors in juvenile rat.

OT mediated by OXTR has been shown to improve social symptoms in autism, reduce anxiety, and induce long-term potentiation in the rodent brain through CREB phosphorylation via activation of c-Raf/MEK/ERK/CREB signaling (Blume et al., 2008; Yoshida et al., 2009). Western blotting results showed that OT treatment remarkably activated the c-Raf/MEK/Erk/CREB signaling in the mPFC of NMS rats via increasing phosphorylation of c-Raf/MEK/Erk/CREB pathway (**Figures 4C–G**, one-way ANOVA followed by the *post hoc* Tukey's tests, $F_{(2,6)Treatment} = 9.655$ [*p*-c-Raf], $P = 0.0133$; $F_{(2,6)Treatment} = 9.480$ [*p*-MEK1/2], $P = 0.0139$; $F_{(2,6)Treatment} = 15.18$ [*p*-Erk1/2], $P = 0.0045$; $F_{(2,6)Treatment} = 17.16$ [*p*-CREB], $P = 0.0033$). Taken together, these data further supported the benefits of OT in improving social symptoms, accompanied by activation of Erk/MAPK cascade.

Expression of Oxytocin Receptor in Neurons Was Decreased in Medial Prefrontal Cortex of Long-Term Neonatal Maternal Separation Rats

Oxytocin receptor, a G protein-coupled receptor that is expressed on the cell membrane, is widely expressed in various tissues, especially in specific regions of the brain, including PFC, hippocampus, hypothalamus, and amygdala, mediating psychological behaviors (Busnelli and Chini, 2018). In neurons, the coupling of OTR with G protein transmits the signal intracellularly, permits OT to affect cell functions, and then causes the activations of intracellular signalings, such as Erk/MEK signaling, and PLC, the effector of phosphoinositide signaling system (Parent et al., 2008; Busnelli and Chini, 2018; Tirko et al., 2018). To further characterize the neuronal cells that express OXTR, we used immunohistochemistry to locate the coexpression of OXTR with different neuronal markers (NeuN for neuron, GFAP for astrocyte, and iBA-1 for microglia) in mPFC. As shown in **Figures 5A–C**, OXTRs were mainly expressed in neurons and astrocytes, a few in microglias. The integrated density of double immunostaining of OXTR/NeuN was significantly decreased in the mPFC of long-term MNS rats compared with control rats, whereas the integrated densities of OXTR in GFAP and Iba-1 were unchanged (two-tailed unpaired *t*-test; $t_{22} = 1.660$ [OXTR/GFAP], $P = 0.1110$; $t_{22} = 7.135$ [OXTR/NeuN], $P < 0.0001$; $t_{22} = 1.194$ [OXTR/Iba-1], $P = 0.2453$).

Low Level of Histone Methylation of Tri-Methylation of Lysine 4 on Histone H3 in Medial Prefrontal Cortex of Long-Term Neonatal Maternal Separation Rats

Recent findings have shown that experiences in early life can epigenetically tune the expression of OXTR, which has been associated with psychiatric disorders and individual differences, including impairments in social recognition and autistic-like behaviors in neural response to social stimuli (Perkeybile et al., 2019). Epigenetic regulatory alteration of OXTR was involved in biological mechanisms underlying the aberrant social behaviors (Tops et al., 2019). Similar findings have indicated that early maternal deprivation in rhesus macaque leads to significantly decreased H3K4me3 (an epigenetic marker for transcriptional activation) binding at OXTR (Baker et al., 2017). Thus, we examined the H3K4me3 level in the mPFC of NMS rats. Our data showed that the H3K4me3 level in NMS rats was markedly downregulated in comparison with the control littermates (**Figures 6A,B**, two-tailed unpaired *t*-test, $t_4 = 4.738$, $P = 0.0091$).

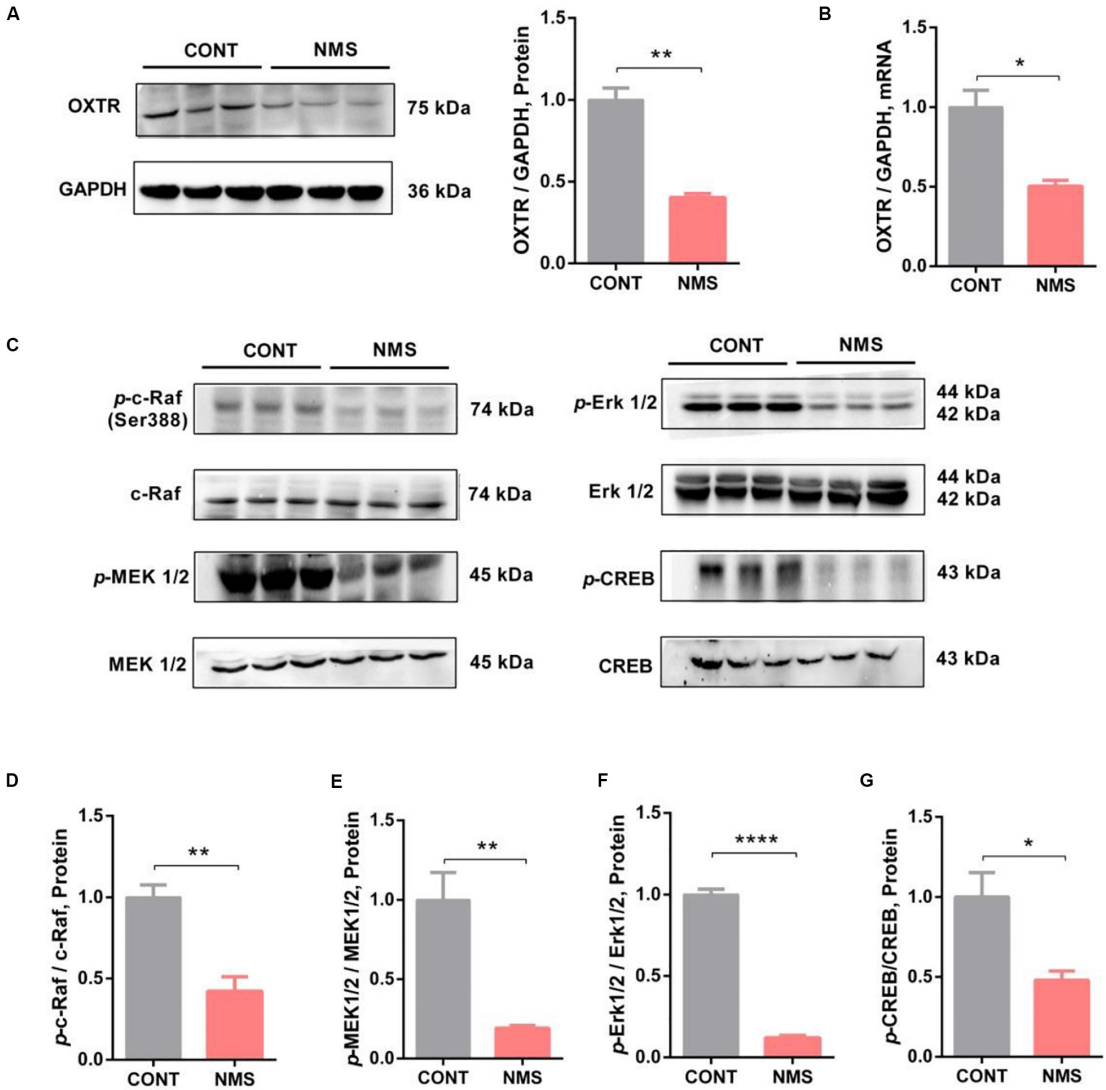

FIGURE 3 | OXTR expression was downregulated with inhibition of Erk/MAPK signaling in long-term NMS rats. **(A)** Immunoblots and quantification analysis of the protein level of OXTR in mPFC from CONT and long-term NMS rats (two-tailed unpaired *t*-test, $t_4 = 7.869$, $P = 0.0014$, $^{**}P < 0.01$). **(B)** Quantitative real-time PCR data on mRNA level of OXTR in PFC from CONT and NMS rats (two-tailed unpaired *t*-test, $t_4 = 4.422$, $P = 0.0115$, $^{*}P < 0.05$). **(C–G)** Immunoblots and quantification analysis of the protein levels of *p*-c-Raf, *p*-MEK 1/2, *p*-Erk 1/2, and *p*-CREB in mPFC from CONT and long-term NMS rats (two-tailed unpaired *t*-test; $t_4 = 4.958$ [*p*-c-Raf], $P = 0.0077$; $t_4 = 4.687$ [*p*-MEK1/2], $P = 0.0094$; $t_4 = 25.46$ [*p*-Erk1/2], $P < 0.0001$; $t_4 = 3.200$ (*p*-CREB), $P < 0.0329$; $^{**}P < 0.01$, $^{****}P < 0.0001$, $^{*}P < 0.05$). Data are represented as mean ± SEM ($n = 3$ in each group).

Histone Demethylases Inhibitor As-8351 Treatment Increased Histone H3K4 Tri-Methylation and Oxytocin Receptor Expression in Medial Prefrontal Cortex of Neonatal Maternal Separation Juvenile Rats and Restored Long-Term Neonatal Maternal Separation-Induced Social Deficits

Early nurture showed the ability to epigenetically tune the expression of OXTR by regulation of the H3K4me3 level (Tops et al., 2019). H3K4me3, serving as an epigenetic marker for transcriptional activation of genes, could provide support for the increase of the OXTR mRNA level, which was regulated by HDMs and HMTs (Greer and Shi, 2012; Miller, 2016; Baker et al., 2017). It was found that inhibition of KDM5B, a critical HDM, could increase OXTR expression by upregulating the H3K4me3 level (Miller, 2016). Herein, to further investigate whether inhibition of HDMs could change NMS stress-induced OXTR expression through epigenetic modulation of histone methylation pathway and then alleviate the impairments in social recognition and autistic-like behaviors, we used a selective KDM5B inhibitor of AS-8351 (Zhang et al., 2019) to treat NMS rats on PND 21–35.

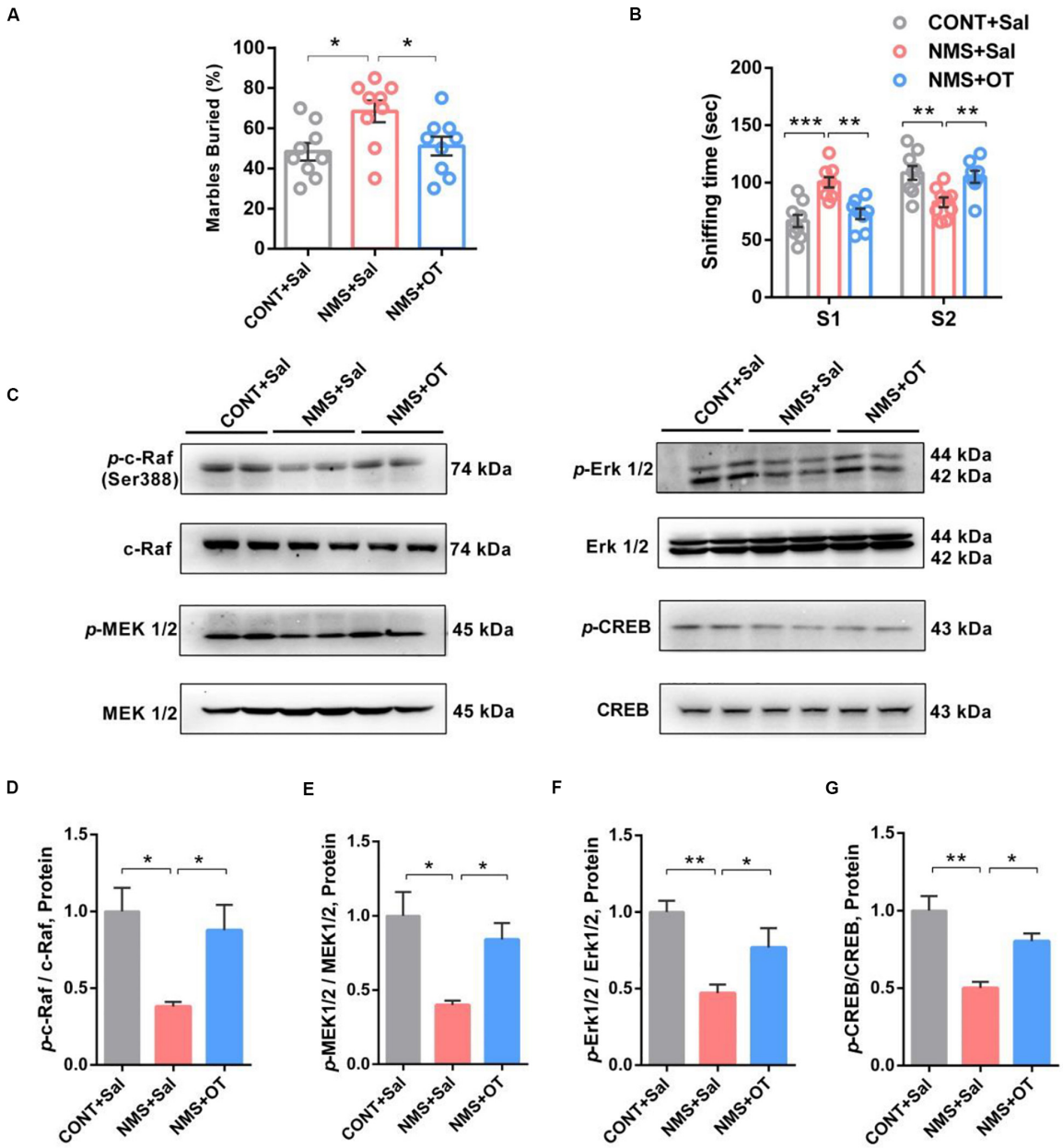

FIGURE 4 | OT treatment rescued impairments in social recognition and autistic-like behaviors in juvenile long-term NMS rats accompanied with activation of Erk/MAPK signaling. **(A)** Marble-burying test, plots showing the percentages of buried marbles after OT treatment (one-way ANOVA followed by the *post hoc* Tukey's tests; $F_{(2,24)Treatment} = 4.983$, $P = 0.0155$, $^{*}P < 0.05$, $n = 9$ in each group). **(B)** Social novelty test, plots showing the time spent investigating either the familiar (S1) or unfamiliar (S2) social rat after OT treatment (two-way ANOVA followed by the *post hoc* Bonferroni's multiple comparison tests; $F_{(2,46)} = 20.50$, $P = 0.0003$, $^{***}P < 0.001$, $^{**}P < 0.01$, $n = 9$ in each group). **(C–G)** Immunoblots and quantification analysis of the protein levels of *p*-c-Raf, *p*-MEK 1/2, *p*-Erk 1/2, and *p*-CREB in mPFC from CONT + Sal., NMS + Sal., and NMS + OT rats after OT treatment (one-way ANOVA followed by the *post hoc* Tukey's tests; $F_{(2,6)Treatment} = 9.655$ [*p*-c-Raf], $P = 0.0133$; $F_{(2,6)Treatment} = 9.480$ [*p*-MEK1/2], $P = 0.0139$; $F_{(2,6)Treatment} = 15.18$ [*p*-Erk1/2], $P = 0.0045$; $F_{(2,6)Treatment} = 17.16$ [*p*-CREB], $P = 0.0033$; $^{**}P < 0.01$, $^{*}P < 0.05$, $n = 3$ in each group). Data are represented as mean ± SEM.

Our studies showed that treatment with AS-8351 could significantly increase the histone methylation of H3K4me3 level (**Figures 6C,D**, one-way ANOVA followed by the *post hoc* Tukey's tests, $F_{(2,6)Treatment} = 5.963$, $P = 0.0375$) and upregulate the OXTR expression both in protein synthesis and mRNA transcription (**Figures 6E–G**, one-way ANOVA followed by the *post hoc* Tukey's tests, $F_{(2,6)Treatment} = 11.04$ [protein], $P = 0.0098$; $F_{(2,6)Treatment} = 10.879$ [mRNA], $P = 0.0101$) in the mPFC of long-term NMS rats. Behavioral results suggested that the NMS treated with AS-8351 showed an increased preference for the stranger rat in the social novelty test (**Figure 6H**, two-way ANOVA followed by the *post hoc* Bonferroni's tests,

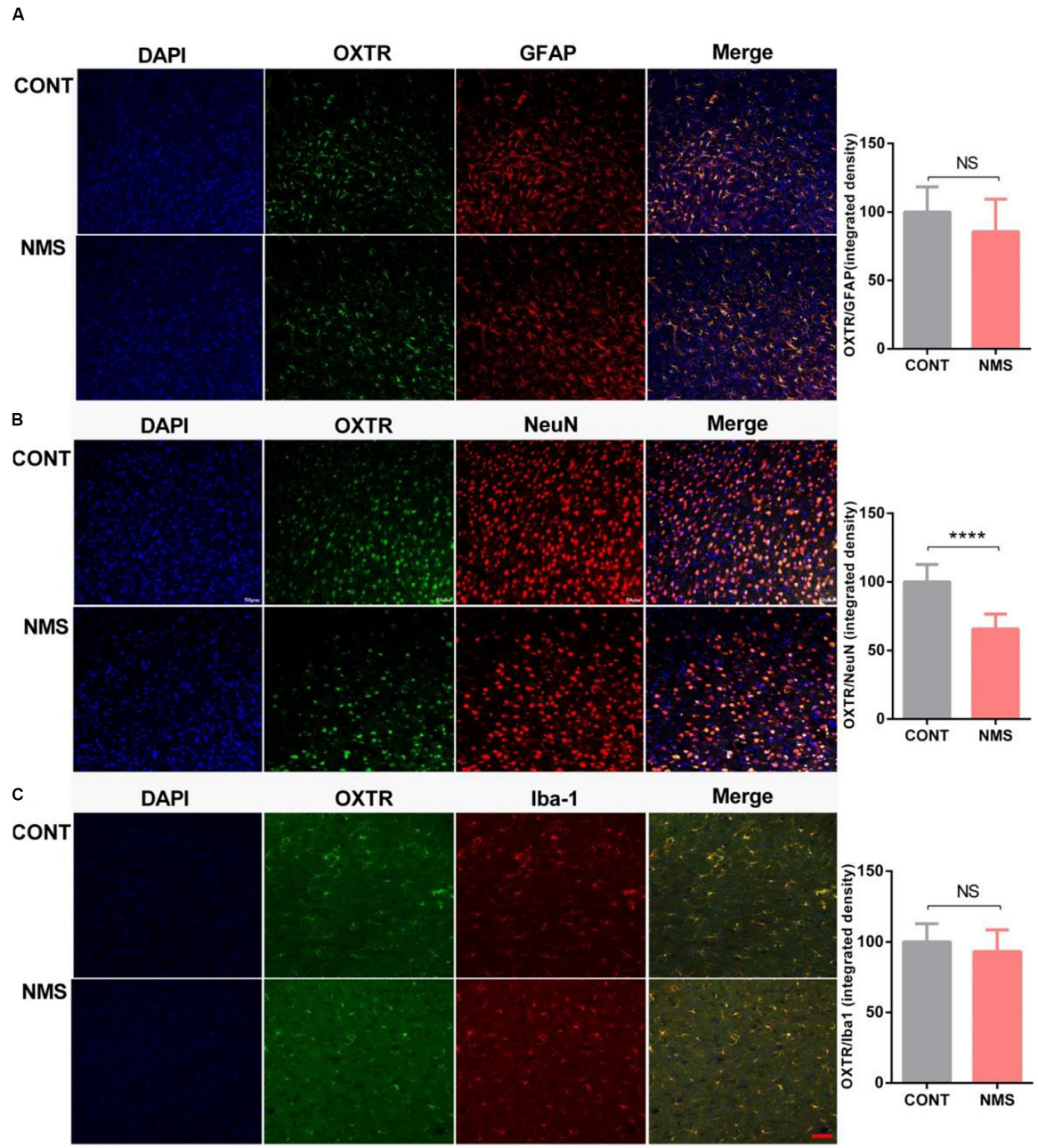

FIGURE 5 | Expression of OXTR in neuronal cells, including neuron, microglia, and astrocyte in mPFC of long-term NMS rats. **(A–C)** Quantification of OXTR positive neural cells (integrated densities; NeuN for neuron marker; iBA1 for microglia marker; GFAP for astrocyte marker) in mPFC slices of different animal groups using immunofluorescence staining. Double immunolabeled surface areas of OXTR/GFAP **(A)**, OXTR/NeuN **(B)**, and OXTR/Iba-1 **(C)** from the mPFC, which are indicated in white lines, were quantified using the ImageJ Program (two-tailed unpaired *t*-test; t_{22} = 1.660 [OXTR/GFAP], P = 0.1110; t_{22} = 7.135 [OXTR/NeuN], $P < 0.0001$; t_{22} = 1.194 [OXTR/Iba-1], P = 0.2453; ****$P < 0.0001$, n.s. $P > 0.05$, n = 3 in each group). Data are represented as mean ± SEM. Scale bars: 50 μm.

$F_{(2,48)}$ = 14.09, $P < 0.0001$) and decreased repetitive and stereotyped behavior in the marble-burying test (**Figure 6I**, one-way ANOVA followed by the *post hoc* Tukey's tests, $F_{(2,24)Treatment}$ = 4.983, P = 0.0155) in comparison with NMS rats. These data indicated administration with the HDM inhibitor rescued the social deficit behaviors observed in NMS subjects via restoring the expression of OXTR.

DISCUSSION

As early life is increasingly recognized as a critical period for brain development and neural plasticity, ELSs during the stage, especially in an infant, may exert long-lasting adverse influences on the brain functions and cause an individual susceptible to psychiatric disorders later in subsequent adolescence and

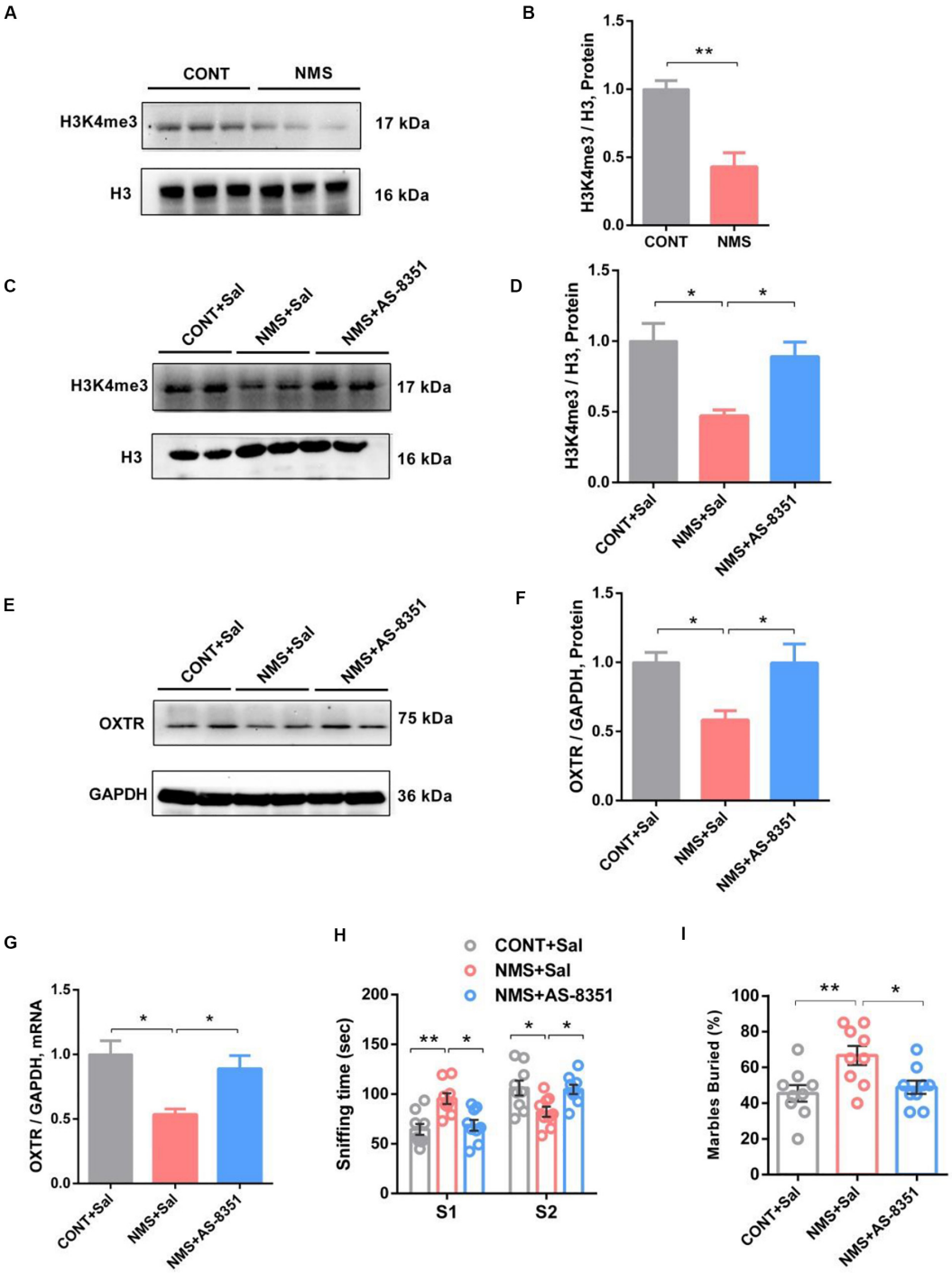

FIGURE 6 | HDM inhibitor treatment restored NMS-induced social deficits with upregulation of H3K4me3 and OXTR in mPFC of long-term NMS rats. **(A,B)** Immunoblots and quantification analysis of the protein levels of H3K4me3 in mPFC from CONT and long-term NMS rats (two-tailed unpaired *t*-test, $t_4 = 4.738$, $P = 0.0091$, $^{**}P < 0.01$, $n = 3$ in each group).). **(C–F)** Immunoblots and quantification analysis of the protein levels of H3K4me3 and OXTR in mPFC from CONT + Sal., NMS + Sal., and NMS + AS-8351 rats after AS-8351 treatment (one-way ANOVA followed by the *post hoc* Tukey's tests; $F_{(2,6)Treatment} = 5.963$ [H3K4me3], $P = 0.0375$; $F_{(2,6)Treatment} = 11.04$ (OXTR), $P = 0.0098$; $^{*}P < 0.05$, $n = 3$ in each group). **(G)** Quantitative PCR showing OXTR mRNA and level in mPFC from CONT + Sal., NMS + Sal., and NMS + AS-8351 rats after AS-8351 treatment (one-way ANOVA followed by the *post hoc* Tukey's tests; $F_{(2,6)Treatment} = 10.87$, $P = 0.0101$; $^{*}P < 0.05$, $n = 3$ in each group). **(H)** Social novelty test, plots showing the time spent investigating either the familiar (S1) or unfamiliar (S2) social rat after AS-8351 treatment (two-way ANOVA followed by the *post hoc* Bonferroni's multiple comparison tests; $F_{(2,48)} = 14.09$, $P < 0.0001$; $^{**}P < 0.01$, $^{*}P < 0.05$, $n = 9$ in each group). **(I)** Marble-burying test, plots showing the percentages of buried marbles after AS-8351 treatment (one-way ANOVA followed by the *post hoc* Tukey's tests; $F_{(2,24)Treatment} = 4.983$, $P = 0.0155$; $^{**}P < 0.01$, $^{*}P < 0.05$, $n = 9$ in each group). Data are represented as mean ± SEM.

adulthood, including anxiety, depression, and impairments in social recognition implicated in ASD (Bevins and Besheer, 2006; Liu et al., 2017). As neonatal stress of disruption in mother–infant contact, NMS results in multiple behavioral changes in rodents, such as depression, anxiety, and visceral hypersensitivity, and increases the risk of disease later in life (Carlyle et al., 2012; Seo et al., 2016; Loewy et al., 2019). In our study, we found that short-term NMS had no effects on general sociability, interest in social novelty, and stereotypical behavior, whereas long-term repetitive NMS could lead to impairments in social recognition and autistic-like behaviors by decreasing the preference for the stranger rat in social novelty and increasing stereotypical behavior in the marble-burying test compared with the littermates, without affecting the learning and memory functions. Furthermore, it did not exclude the possibility that the starting time of stress might also affect behavioral phenotypes. The early postnatal stage, specifically PND 4 to PND 14 in rats, is crucial for the development of the stress system and is considered as the stress hyporesponsive period (Schmidt et al., 2003). As the two paradigms started at different PND, this brought about an assumption whether another short-term NMS (starting from PND 1 to 7) would cause impairments in behaviors observed in the long-term NMS (PND 1–20), which was indeed a limitation of this study. Thus, it might be concluded that the duration, severity of stress, and starting time of stress might be implicated in the behavioral phenotypes.

It was found that long-term repetitive NMS caused significant weight loss, which might also trigger behavioral changes in particular when the animals were under a subinflammatory state (Rideau Batista Novais et al., 2016; Mairesse et al., 2019). Maternal deprivation was severe psychosomatic stress that could induce many abnormal changes besides weight loss. Even the NMS rats showed significant weight loss during adolescence; they also displayed other normal behaviors, including memory recognition and sociability, except for impairments in social novelty and stereotyped behavior. We considered that weight loss was part of the consequences that resulted from maternal deprivation due to the neonatal stress, which might not be the primary cause of the behavioral deficits but an inevitable result of neonatal maternal deprivation that led to a nutritional deficiency during the stress.

Exposure to NMS, serving as an early life adverse event, showed the potentiality of increasing individual vulnerability to later psychiatric disorders in subsequent life and led to impairments in social recognition and autistic-like behaviors (Kambali et al., 2019). Our findings also showed that long-term repetitive NMS induced deficits in the development of the social system. However, the underlying molecular mechanism has not largely been elucidated. Related studies suggested that early experiences, including the quality and extent of maternal care, exerted long-lasting alterations in social behaviors, anxiety, and fearfulness, along with aberrant HPA axis activity and the expression of brain-derived neurotrophic factor (Levine, 1970; Meaney, 2001; Daniels et al., 2004; Seo et al., 2016; Liu et al., 2017). Early rearing history had an effect on OXTR expression (Baker et al., 2017), which was consistent with our findings, indicating that disruption in maternal care in rats resulted in remarkable downregulation of OXTR repression in mPFC, a key brain region that played an essential role in mediating social behavior (Amodio and Frith, 2006). OXTR is widely expressed in different functional brain regions, including PFC, hippocampus, amygdala, and hypothalamus, exerting important roles in the regulation of complex social behaviors, as well as in psychiatric disorders characterized by social deficits (Bakermans-Kranenburg and van Ijzendoorn, 2014). OT is released in the paraventricular nucleus and supraoptic nucleus of the hypothalamus in the brain and also implicated in regulating the social behaviors besides mating, maternal behavior, attachment, aggression, and sexual behavior. Recent studies have demonstrated that OT produces anxiolytic, antidepressant, and pro-social effects in animal models (Ferguson et al., 2001; Heinrichs et al., 2003; Sharma et al., 2019). Due to its functions on the improvement of social recognition, the intranasal administration of OT has been under clinical studies for the treatment of ASD characterized by social deficits in adults and juveniles (Anagnostou et al., 2012). Studies also demonstrated that OXTR variants were involved in children subjected to emotional neglect, and deficits in OXTR or OT expression could result in pervasive social deficits in mice (Takayanagi et al., 2005; Womersley et al., 2019). OT/OXTR system is crucial in the regulation of social behaviors; as a result, NMS-induced OXTR downregulation may be implicated in the impairments of social recognition and autistic-like behaviors. The actions of both central and peripheral OTs are mediated through OXTR. As a G protein-coupled receptor that can receive and then transduce information into cells via signaling pathways, OXTR is expressed on the neural cell membrane in the brain, such as neuron and astrocyte (Hidema et al., 2016). The integrated density of OXTR on neurons by double immunostaining was significantly reduced, which might affect the functions of neurons. The main signaling pathways activated by the OXTR in neuronal cells are closely related to the intracellular effects and contribute to the qualitative and quantitative outcomes of OT responses in the brain. It has been reported that c-Raf/MEK/Erk/CREB (Erk-MAPK) signaling cascade functions as the intracellular downstream pathway of OT/OXTR (Tomizawa et al., 2003). Moreover, Neumann et al. (2013) demonstrated that OXTR/Erk/MAPK signaling mediated OT anxiolytic effects via activating the phosphorylation (Blume et al., 2008; Jurek et al., 2012; van den Burg et al., 2015). Besides the downregulation of OXTR, our data showed that the OXTR downstream signaling of Erk/MAPK cascade was remarkably inhibited in the PFC of NMS rats, which might be involved the downregulation of genes and the ELS-induced impairments of behaviors or the reactiveness in the response of stresses. OT has quite a limited ability to penetrate the blood–brain barrier and can only rely on related receptor transport (Leng and Ludwig, 2016; Duarte-Guterman et al., 2020), which is the reason that the current clinical administration is mainly through intranasal administration. In contrast, it has been reported that peripheral OT administration improves not only depressive-like behavior but also object recognition and social recognition in high fat diet-fed mice and can increase OT mRNA expression in the hypothalamus OT (Hayashi et al., 2020). Moreover, peripheral delivery of OT could lead to the central release of endogenous

OT (Neumann et al., 2013). After i.p. injection of OT, NMS rats showed improved social deficient behavior accompanied by activation of Erk/MAPK signaling, indicating that inhibition of OXTR/Erk/MAPK signaling might be involved in NMS-induced social impairments. Furthermore, there were papers reporting that epigenetic variations, including DNA methylation, histone methylation, and acetylation, were involved in environmental exposure and psychiatric disease burden resulting from persistent disruptions of mother–infant interactions (McGowan et al., 2009; Tops et al., 2019). Early life adverse experiences such as childhood abuse influenced HPA function and led to aberrant behaviors in rats through epigenetic programming of gene expression (McGowan et al., 2009). A similar study showed that disruptions of mother–infant interactions in rhesus macaques resulted in a dramatic decrease of OXTR mRNA via epigenetic regulation of decreased H3K4me3, which contributed to the behavioral pathology observed in rhesus macaques that are subjected to early maternal deprivation (Baker et al., 2017). Epigenetic variability in the human OXTR gene caused by environmental events, especially those occurring during early childhood, was associated with the alteration of social sensitivity (Kraaijenvanger et al., 2019). In the prairie vole, early nurture could epigenetically tune the OXTR, which was associated with individual differences in neural response to social stimuli and impairments in social behavior (Perkeybile et al., 2019). Recently, it was reported that maternal deprivation in rhesus macaque caused alterations of H3K4me3 level binding at genes using a ChIP-sequencing assay and that the most robust gene is OXTR, which was correlated with the phenotypes of separation anxiety and arousal observed (Baker et al., 2017). NMS rats showed an epigenetic down-modulation of OXTR with a decreased H3K4me3 level in mPFC. H3K4me3, a marker for transcriptional activation, could provide support for the increase of the OXTR mRNA transcriptional level by facilitating the transcription factor to bind to the OXTR promoter (Mackowiak et al., 2014; Dincer et al., 2015; Baker et al., 2017; Varma et al., 2017). H3K4me3 level is mediated by HDMs and HMTs (Kouzarides, 2007). Inhibition of HDMs, which serve as a main regulatory factor of H3K4me3, could increase OXTR expression via upregulating H3K4me3 (Miller, 2016). Our results showed that NMS rats administrated with AS-8351, a potent inhibitor of HDMs, presented an increase of H3K4me3 OXTR levels in mPFC and restored social behaviors. Those findings indicated that disruptions of mother–infant interactions had an epigenetic effect on OXTR expression via regulation of H3K4me3, which involved NMS-induced impairments in social recognition and autistic-like behaviors.

CONCLUSION

Early life stresses, especially during the neonatal or infant period, significantly increase the susceptibility of mental illness in juveniles and adult individuals and may lead to impairments in social recognition and autistic-like behaviors in subsequent life. Here, NMS in rats was used as a stress model of disruption in mother–infant contact. Our behavioral results showed that juvenile rats subjected to short-term NMS did not induce autistic-like behavioral deficits in social novelty, social approach, and marble-burying tests. In contrast, long-term NMS stress (PND 1–20) triggered impairments in social recognition or memory repetitive and stereotypical autistic-like behavior in juvenile MNS rats compared with the control littermates without affecting the learning and memory functions in the novel object recognition test. The molecular mechanism study underlying the NMS-induced social deficits showed that OXTR expression in the mPFC of NMS rats was evidently downregulated, especially in neurons, along with the inhibition of its downstream signaling of the Erk/MAPK cascade. OT treatment could rescue NMS-induced behavioral deficits with phosphorylation activation of the Erk/MAPK cascade. Moreover, our findings showed that NMS rats exhibited a low level of H3K4me3, which is involved in promoting the expression of OXTR. Treatment with the inhibitor of H3K4 demethylase alleviated the abnormal behaviors in NMS rats and increased the expression of OXTR in mPFC. Those findings identified a mechanism by which repetitive long-term disruption in mother–infant contact influenced later displays of social and autistic-like behaviors and suggested an epigenetic tuning of OXTR for NMS stress.

DATA AVAILABILITY STATEMENT

The data from the present study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by Experimental Animal Committee of Shanghai Jiao Tong University School of Medicine.

AUTHOR CONTRIBUTIONS

JC, JW, and Y-XW conceived and designed the experiments. JW, LM, BY, and PJ performed the experiments. JC and JW analyzed the data. JW, JC, and Y-XW preparation of the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Effect of Early Life Stress on the Epigenetic Profiles in Depression

Ming Li, Xiying Fu, Wei Xie, Wanxu Guo, Bingjin Li, Ranji Cui and Wei Yang**

Jilin Provincial Key Laboratory on Molecular and Chemical Genetic, Second Hospital of Jilin University, Changchun, China

Depression is one of the most common mental disorders and has caused an overwhelming burden on world health. Abundant studies have suggested that early life stress may grant depressive-like phenotypes in adults. Childhood adversities that occurred in the developmental period amplified stress events in adulthood. Epigenetic-environment interaction helps to explain the role of early life stress on adulthood depression. Early life stress shaped the epigenetic profiles of the HPA axis, monoamine, and neuropeptides. In the context of early adversities increasing the risk of depression, early life stress decreased the activity of the glucocorticoid receptors, halted the circulation and production of serotonin, and reduced the molecules involved in modulating the neurogenesis and neuroplasticity. Generally, DNA methylation, histone modifications, and the regulation of non-coding RNAs programmed the epigenetic profiles to react to early life stress. However, genetic precondition, subtypes of early life stress, the timing of epigenetic status evaluated, demographic characteristics in humans, and strain traits in animals favored epigenetic outcomes. More research is needed to investigate the direct evidence for how early life stress-induced epigenetic changes contribute to the vulnerability of depression.

Keywords: early life stress, epigenetic, depression, DNA methylation, HPA axis

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Fushun Wang,
Nanjing University of Chinese Medicine, China

Reviewed by:
Wenbo Luo,
Liaoning Normal University, China
Yiqun Wang,
Fudan University, China

****Correspondence:***
Wei Yang
wyang2002@jlu.edu.cn
Ranji Cui
cuiranji@jlu.edu.cn

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INTRODUCTION

Depression is one of the most common mental health problems worldwide. According to a report from the Global Burden Data, the mental disorder was on the list of the leading causes of death (GBD 2017 Causes of Death Collaborators, 2018). Hitherto, though depression is characterized by a disturbance of the hypothalamic-pituitary-adrenal (HPA) axis, monoamine or neuroplasticity, and neurogenesis, how these biological events react to environment changes has not been fully illustrated (Malhi and Mann, 2018). Epigenetics is the study of heritable and genetic events added to traditional genetics. Epigenetics aims to explore the alterations of DNA methylation (DNAm), histone modification, and non-coding RNAs. In other words, epigenetic changes do not alter the original DNA sequence and it perfectly explains the interaction between the environment and genetics (Dupont et al., 2009). Early life stress (ELS) is a prevalent experience that happens before birth or in early postnatal life. On the one hand, ELS increased the risk of depression or worsened depressive symptoms. On the other hand, ELS caused epigenetic changes in the HPA axis, serotonin, dopamine, and some neuropeptides (McGowan et al., 2009; Roth et al., 2009; Bai et al., 2012; Kang et al., 2013; Ouellet-Morin et al., 2013; Zhang et al., 2015; Bahi, 2016; Bustamante et al., 2016; Tyrka et al., 2016; Williams et al., 2016; Wang et al., 2017). Therefore, epigenetic events bridge the connection between ELS and depression and some studies have proven

the effect of epigenetic changes on the accumulating risk of developing depression in those who were subjected to ELS (Kang et al., 2013; Melas et al., 2013; Wankerl et al., 2014; Cecil et al., 2016; Tyrka et al., 2016; Dickson et al., 2018; Opel et al., 2019). However, though being exposed to ELS increased depressive incidents, not all individuals who suffered ELS acquired depression. Genetic precondition determines whether exhibited pro-depressive epigenetic alteration or stress-resistant profiles confronted ELS. Moreover, subtypes of ELS, gender, exposed timing, and duration matter in the role of ELS in depression (Cecil et al., 2016; McCoy et al., 2016; Frodl et al., 2017; Reus et al., 2017). In this system review, we will discuss the effect of ELS on epigenetic alterations, which mainly facilitated the occurrence and development of depression in two independent sections, and in each section, we will discuss DNAm, histone modification, and RNA transcripts.

OVERVIEW OF EPIGENETICS

Epigenetic events mainly refer to the alterations of DNAm, histone modification, and microRNA (miRNA) (Dupont et al., 2009). Among them, DNAm is the most well-defined and thoroughly investigated (Ouellet-Morin et al., 2013; Montirosso et al., 2016; Tyrka et al., 2016; Janusek et al., 2017; Wikenius et al., 2019). DNAm mostly occurs in CpG sites. Generally, most cytosine–phosphate–guanine (CpG) islands (CG enriched) remained hypomethylated but the other CpG sequences remained hypermethylated. Methylation or demethylation potentially suppresses or promotes gene transcription, respectively (McGowan et al., 2009; Melas et al., 2013; Ouellet-Morin et al., 2013; Peng et al., 2018). Secondly, histone modification includes acetylation, methylation, ubiquitylation, phosphorylation, SUMOylating, ribosylation, and citrullination. Among them, acetylation is the most highly studied in mental disorders (Levine et al., 2012; Seo et al., 2016; Wang et al., 2018; Karen and Rajan, 2019). Histone modification can loosen or tighten the chromosome to alter transcription. miRNAs can directly or indirectly regulate genes and miRNAs were also upregulated or downregulated in depressed patients who had ELS (Dupont et al., 2009).

DEFINITION OF DEPRESSION

Depression is a prevalent mental illness and it leads to an increased health burden worldwide (GBD 2017 Causes of Death Collaborators, 2018). In a hypothesis that described the progression of depression, the HPA axis was most prominent. The HPA axis plays a critical role in the response to stress, and a disturbance of the HPA axis results in vulnerability for depression (Bjorkenstam et al., 2017; Reus et al., 2017). It is well-documented that ELS enhanced the vulnerability for depression (Kim et al., 2013; Culpin et al., 2015; St Clair et al., 2015; Airagnes et al., 2016; Williams et al., 2016; Bjorkenstam et al., 2017; Dahl et al., 2017; Opel et al., 2019; Tracy et al., 2019). Depressed subjects may bear the increased level of plasma cortisol of excessive stress-induced and impaired functions of the glucocorticoid receptors (GR) (Malhi and Mann, 2018). Excessive cortisol and impaired GRs increased the susceptible response to stress. However, some depressed patients did not obtain cortisol changes and therefore other biomedical alternations may also account for the occurrence of depression. Serotonin or 5-hydroxytryptamine (5-HT), noradrenaline, and dopamine are monoamines involved in the progression of depression. Selective serotonin reuptake inhibitors regulate the synaptic plasticity, improving depressive symptoms in depressed patients (Kinnally et al., 2011; Kang et al., 2013; Ouellet-Morin et al., 2013). Besides, some neuropeptide-modulated neurogenesis and neuroplasticity. Their alterations increased the risk for depression and worsened the response to antidepressants, of which, the brain-derived neurotrophic factor (BDNF) was heatedly investigated (Roth et al., 2009; Bai et al., 2012; Doherty et al., 2016). Besides the hypothesis, pro-inflammation was presumed to deteriorate or even accelerate the progression of depression (Janusek et al., 2017). Pro-inflammatory status was found among the depressed and possibly the pro-inflammation interacted with the dysfunctional HPA axis (Malhi and Mann, 2018). Regarding the mechanism of depression, genetic factors cannot be ignored because of the disparity among populations and sex in dealing with stress (Perroud et al., 2011). Epigenetic profiles are the internal temperament that defines external personal characteristics. Epigenetic changes may be long-lasting (Dickson et al., 2018; Roberts et al., 2018). ELS happens in a prenatal or early postnatal time frame but is attributed to the personal susceptibility to depression (St Clair et al., 2015; Bjorkenstam et al., 2017; Reus et al., 2017). Therefore, ELS is the perfect example to better explain the epigenetic role in the evolution of depression.

EPIGENETIC STUDIES FOCUSED ON HUMAN BEINGS

Robust clinical studies suggested that ELS increased the risk of depression. Epigenetic modification underlies the regulation of the HPA axis, monoamine, and neuropeptides. The epigenetic studies were either conducted in a site-specific or genome-wide pattern. Of those studies, DNAm was richly demonstrated. Firstly, we introduce epigenetic regulation focused on interesting regions. McGowan and his colleagues found that the neuron-specific glucocorticoid receptor (NR3C1) promoter was hypermethylated in the postmortem hippocampus of suicide victims with a history of childhood abuse compared to those victims without child abuse (McGowan et al., 2009) and increased DNAm of the exon 1F NR3C1 promoter was also observed in the peripheral blood of patients with major depressive disorders (MDD) who suffered ELS (Perroud et al., 2011). The patch hypermethylation within the NR3C1 promoter resulted in the decreased expression of NR3C1 (McGowan et al., 2009; Perroud et al., 2011). Furthermore, the severity and the number of types of ELS positively correlated with the exon 1F NR3C1 DNAm in MDD adults (Perroud et al., 2011). Except for childhood adversities, prenatal distress also modulated the DNAm of the exon 1F NR3C1 promoter of newborns. Prenatal depressive symptoms increased the exon 1F

NR3C1 DNAm in male infants rather than in female infants (Braithwaite et al., 2015). Likewise, the composite measurement of child maltreatment was positively correlated with the DNA methylation of exons 1D and 1F in the promoter of the GR gene in preschool children (Tyrka et al., 2015). Moreover, compared with suicides without childhood abuse, the exon 1B and 1C, but not 1H GR promoters were also hypermethylated while the expression of the variants 1B and 1C were decreased in the hippocampus of suicides with ELS exposure (Labonte et al., 2012). Additionally, in a study of monozygotic twin pairs, Palma-Gudiel et al. (2018) demonstrated that increased exon 1D NR3C1 CpG-specific methylation was related to depressive symptoms and decreased hippocampal connectivity. Moreover, another monozygotic twin study revealed that DNAm at the NR3C1 mediated the association between childhood trauma and depression (Peng et al., 2018). In short, the publications we mentioned above supported that fact that an increased methylated GR gene or regions in individuals with ELS and the level of DNAm positively correlated with the severity of ELS or specific subgroups of ELS. The hypermethylation of GR genes likely dysregulated the HPA axis and made the subject more vulnerable to stress and possibly more likely to develop depression. In contrast to this opinion, Tyrka et al. (2016) proposed that reduced DNAm of the NR3C1 was associated with childhood maltreatment and depressive disorders in adults. In this study, ELS and current or post depressive or anxiety disorders were correlated with a reduction in exon 1F NR3C1 promoter methylation either at individual CpG sites or across the gene. Moreover, the altered NR3C1 DNAm induced a blunt cortisol response to stress (Tyrka et al., 2016). Likewise, Alexander et al. (2018) found an increased DNAm of the NR3C1 exon 1F promoter in healthy adults with childhood trauma exposure. However, this change seemed independent of the cortisol response to stress in those unexposed and mildly or moderately ELS-exposed individuals (Alexander et al., 2018). What is more, one study that enrolled healthy women aged 40 + assumed no significant associations between childhood adversity and DNAm in the NR3C1 promoter but higher DNAm in the ERα shore correlated with higher levels of adversity (Fiacco et al., 2019). In addition, childhood maltreatments and MDD induced different epigenetic changes at the NR3C1 promoter. Specifically, childhood maltreatments were associated with increased DNAm in an EGR1 transcription factor binding site (NGFI-A), whereas MDD was associated with a decrease in DNA downstream of NGFI-A. It was only childhood maltreatments, rather than the depressive symptoms, that were associated with reduced NR3C1 expression (Bustamante et al., 2016). Therefore, though most studies supported that ELS increased the NR3C1 DNAm and possibly that the epigenetic alterations resulted in a blunt HPA axis, emerging evidence indicated that even ELS altered NR3C1 DNAm, this molecular change may not be powerful enough to develop depression (Tyrka et al., 2016; Alexander et al., 2018; Fiacco et al., 2019). It was reported that depressive patients harbored different profiles of the HPA axis, which might be due to a feature of the NR3C1 DNAm (Malhi and Mann, 2018). Furthermore, demographic characteristics (i.e., age and gender), types and severity of ELS (physical abuse, sexual abuse, emotional abuse, physical and emotional neglects), tissue specificity (i.e., subregions of the brain, blood), dynamic changes in epigenetic profiles (interacting with continuous environment actions), and study design contributed to this discrepancy (McGowan et al., 2009; Perroud et al., 2011; Kember et al., 2012; Bustamante et al., 2016; Cecil et al., 2016; Tyrka et al., 2016; Alexander et al., 2018; Palma-Gudiel et al., 2018; Peng et al., 2018; Fiacco et al., 2019). Besides, because research focusing on the figure of histone modification and non-coding RNA within NR3C1 is lacking, it is unknown whether DNAm, histone modification, and non-coding RNA coordinate or disintegrate with each other. Therefore, ELS probably altered the epigenetic profile of the NR3C1, but whether this change mediated the association between ELS and depression is in debate. ELS reshaped the epigenetic figures of the HPA axis, especially NR3C1, and mediated the cortisol response to the stress (McGowan et al., 2009; Perroud et al., 2011; Tyrka et al., 2016; Palma-Gudiel et al., 2018). Increased NR3C1 DNAm was assumed to downplay the function of GRs and exert a blunt cortisol level in response to stress (Palma-Gudiel et al., 2018; Peng et al., 2018).

Further, except for the evidence focused on the epigenetic alterations of the GR genes, the genes involved in the regulation of the HPA axis were also explored. FKBP5 (FK506 binding protein 5), the functional glucocorticoid response element, encodes the protein that interacts with corticoid receptors. Elevated glucocorticoids activate the expression of FKBP5 or inversely, the elevated FKBP5 reduces the activity of GRs. Klengel et al. (2013) proposed that allele-specific FKBP5 DNA demethylation mediated gene–childhood trauma interactions. The demethylation event resulted in long-term dysregulation of the stress response and therefore increased the risk of developing stress-related psychiatric disorders in adulthood (Klengel et al., 2013; Tozzi et al., 2018). Moreover, according to a study of epigenome-wide blood DNAm, the DNAm of the kit ligand gene (KITLG) strongly medicated the relationship between childhood trauma and cortisol stress reactivity in humans (Houtepen et al., 2016). Therefore, with regard to the clinical investigation, except for the evidence of directly modified GRs, changes in the genes that interacted with GRs were also observed. It is possible that the genetic background, i.e., polymorphism, modulated the function of the GRs and their binding or responding elements when individuals were exposed to ELS and the subsequent risk of depression (Klengel et al., 2013; Houtepen et al., 2016; Peng et al., 2018; Tozzi et al., 2018).

Regarding the epigenetic changes of the 5-HT genes, SLC6A4 (solute carrier family 6 member 4) encodes the protein that transports the neurotransmitter serotonin from synaptic spaces into presynaptic neurons. MAO-A (monoamine oxidase A) encodes the enzymes that catalyze the monoamine, such as dopamine, norepinephrine, and serotonin. In other words, MAO-A was designed to eliminate monoamine. Therefore, SLC6A4 terminates the synaptic actions of serotonin. Current evidence supported that ELS was correlated with increased SLC6A4 DNAm and hence reduced the level of SLC6A4

(Kang et al., 2013; Ouellet-Morin et al., 2013; Wankerl et al., 2014). It is likely though that decreased SLC6A4 briefly accumulated the 5-HT in the synaptic cleft, the activated MAO-A enhanced the clearance in the absence of 5-HT recycle (Melas et al., 2013). Therefore, it is the increased SLC6A4 DNAm and decreased MAO-A that synergistically lessens the normal function of 5-HT. Therefore, weak 5-HT activity can be associated with depressive symptoms. Wang et al. suggested that childhood physical aggregation increased SLC6A4 DNAm in peripheral white blood cells and hypermethylated CpG sites reflecting serotonin synthesis in human brains (Wang et al., 2012). Kang and colleagues addressed that the hypermethylation of the SLC6A4 promoter was associated with childhood adversities and worse clinical presentations of 108 depressive patients. However, the methylation status was not correlated with treatment outcomes (Kang et al., 2013). Moreover, a longitudinal study of discordant monozygotic twins demonstrated that increased SLC6A4 DNAm was associated with bullying victimization. Children with a higher SLC6A4 DNAm level had impaired glucocorticoid-medicated feedback and it was assumed that this was linked to depression (Ouellet-Morin et al., 2013). Moreover, this study revealed that even patients free of bullying events, increased methylation level was observed at the age of 5 years, while this increasing trend vanished at the age of 10 in non-bullied patients. Whereas in the group of bullying victimization, higher SLC6A4 DNAm was still noticed at the age of 10 compared with the non-bullied monozygotic twins across the same time frame (Ouellet-Morin et al., 2013). Furthermore, compared with full-term infants, greater socio-emotional stress was associated with increased SLC6A4 promoter region methylation in 3-month-old very preterm infants (Montirosso et al., 2016). Moreover, very preterm infants were exposed to more stress in the very early stages of life and the subsequent alteration of SLC6A4 DNAm correlated with a reduced anterior temporal lobe (Fumagalli et al., 2018). Concerning female adults who harbored the allele MAOA-L and were exposed to childhood adversities concurrently, depression was more likely to develop. These females obtained decreased MAO-A DNAm and epigenetic modification was negatively correlated with childhood adversities (Melas et al., 2013). However, Wankerl et al. illustrated that maternal prenatal stress and child maltreatment were associated with reduced SLC6A4 mRNA expression, but the declination of SLC6A4 mRNA level was not likely mediated by the DNAm of the CpG island of the gene in healthy young adults (Wankerl et al., 2014). Therefore, though healthy individuals were subjected to early tragedies, the epigenetic mediation in these healthy persons helped to reduce the risk of developing depression.

Apart from the HPA axis and serotonin system, epigenetic regulation also presented at inflammation sites and in some neuropeptides. Depression often presents pro-inflammation and decreased neurogenesis. As for the pro-inflammatory profile, it was reported that elevated pro-inflammatory factors were associated with childhood adversities. Janusek et al. (2017) reported that the reduced DNAm of the IL-6 promoter was associated with childhood trauma in adult African American men. The reduced IL-6 promoter was accompanied by an increased acute stress-induced IL-6 response, and a blunted cortisol response. On the other hand, ELS seemed to contribute to the increased DNAm of BNDF in individuals with ELS, and this epigenetic alteration was long-lasting and possibly impacted the offspring of the females (Braithwaite et al., 2015; Peng et al., 2018). Furthermore, the functional single nucleotide polymorphisms (SNPs) of BDNF were moderated by the DNAm values at BDNF specific promoter sites and the interaction between SNPs and DNAm enhanced the susceptibility to depression (Ferrer et al., 2019). Therefore, current research suggested that ELS could alter the epigenetic profiles to present pro-inflammatory functions and reduce neurogenesis. However, more investigations should be conducted to prove that epigenetic alterations are associated with the risk of depression.

Apart from the research that were directly linked to the previously mentioned site-specific epigenetic changes, genome-wide studies were also performed. It was reported that in a group of French-Canadian men with severe childhood abuse, 362 promoters were differently methylated, and some involved in the neural plasticity (Labonté et al., 2012). Likewise, Suderman et al. (2014) revealed that 997 gene promoters were differentially methylated in adult men exposed to childhood abuse. Of those regulated genes, some involved in the key cell pathways were associated with transcriptional regulation and development. Moreover, ELS was associated with different methylated values at 2868 CpG sites in maltreated children. However, genes were involved in the epigenetic changes linked largely to non-mental disease (Yang et al., 2013). Contrasting with the epigenetically altered genes in maltreated children, maltreated young adults obtained several stress-related epigenetic genes. Besides, Cecil et al. demonstrated that different subtypes of ELS correlated with various epigenetic characters (Cecil et al., 2016). As for the possibility of maternal mental health affecting the epigenetic figure of their offspring, Roberts et al. (2018) suggested that exposure to childhood abuse was associated with human sperm DNAm. However, a longitudinal genome-wide study suggested no significant genome-wide association between maternal depressive symptoms and infant DNAm (Wikenius et al., 2019). Therefore, with regard to the genome-wide epigenetic alteration, variable profiles were more noticeable. Possibly the evaluated timing, subtypes of childhood adversities were closely correlated with the epigenetic profiles.

All in all, with respect to the clinical investigation of the ELS-induced epigenetic alterations, the increased DNAm of the NR3C1 promoters, allele-specific FKBP5 DNA demethylation, the hypermethylation of the SLC6A4 promoters, and the increased DNAm of BNDF were associated with ELS. These epigenetic alterations were assumed to increase the risk for depression, and these transformations lasted long into older adulthood or even impacted the offspring (McGowan et al., 2009; Kang et al., 2013; Ouellet-Morin et al., 2013; Wankerl et al., 2014; Montirosso et al., 2016; Tyrka et al., 2016; Fumagalli et al., 2018; Palma-Gudiel et al., 2018; Peng et al., 2018; Roberts et al., 2018; Fiacco et al., 2019; Wikenius et al., 2019). However, regarding individuals who suffered ELS but remained healthy across adulthood, ELS may bring about different or even opposite

TABLE 1 | Early life stress modulates epigenetic profiles in human.

Participants	Gender	Types of ELS	Measured methods	Epigenetic outcome	Phenotypic bridge	References
Healthy adults	62% F	PA, SA, EA, PN, EN	Pyrosequencing	Glucocorticoid receptor promoter DNAm in leukocyte↓	Healthy adults exposed to ELS differ in epigenetic profile to lifetime depressed patients	Tyrka et al., 2016
Healthy young adults	50% F	PA, SA, EA, PN, EN	Pyrosequencing	1_F NR3C1 promoter DNAm↑	No direct association between ELS and NR3C1-1_F DNAM	Alexander et al., 2018
Infants at 2 months of age	M + F	Prenatal depressive symptoms	Pyrosequencing	1_F NR3C1 promoter DNAm↑ in male infants; IV BDNF promoter DNAm↓ in both gender	No association between maternal cortisol and infant DNA methylation	Braithwaite et al., 2015
Preschool children at the age of 3–5 years	61%F	PA, SA, EA, PN, EN	Pyrosequencing	1_D and 1_F NR3C1 promoter DNAm↑	N/A	Tyrka et al., 2015
MDD adults	62%F	Childhood maltreatment (mixed types)	Pyrosequencing	Childhood maltreatment associated with glucocorticoid receptor promoter DNAm in peripheral blood↑	Childhood maltreatment other than depression correlated with NR3C1 expression	Bustamante et al., 2016
Suicide victims	M	Childhood abuse/neglect	Sodium bisulfite mapping	1_F NR3C1 promoter DNAm↑	NR3C1 expression in hippocampus↓	McGowan et al., 2009
Suicide victims	M	Severe childhood abuse	Immunoprecipitation + microarray	248 hypermethylated and 114 hypomethylated	N/A	Labonté et al., 2012
British adults	M	PA, EA, SA	Methylated DNA immunoprecipitation + pyrosequencing	311 hypermethylated and 686 hypomethylated	N/A	Suderman et al., 2014
Young adults	53% F	PA, SA, EA, PN, EN	Microarray	Stress-related genes differently methylated	Possible increased risk for psychiatric and physical disorders	Cecil et al., 2016
Healthy adults	M	Physical aggression	Pyrosequencing	SLC6A4 promoter DNAm↑	5-HT synthesis↓	Wang et al., 2012
MDD patients	81% F	Parental loss, financial hardship, physical and sexual abuse	Pyrosequencing	SLC6A4 promoter DNAm↑	Worse depressive symptoms	Kang et al., 2013
Very preterm and full-term infants at the age of 3 months	44% F	Socio-emotional stress	Pyrosequencing	SLC6A4 promoter DNAm↑ in very preterm infants	Worse negative emotion	Montirosso et al., 2016
Monozygotic twins	Same-sex within twins	Bullying victimization	Amplified site-specific and evaluated via Sequenom EpiTYPER system	SERT promoter DNAm↑	Blunted cortisol responses to stress	Ouellet-Morin et al., 2013
Healthy young adults	50% F	Maternal prenatal stress/child maltreatment	Pyrosequencing	No significant DNAm alterations associated with ELS	Lower SERT mRNA levels	Wankerl et al., 2014
African American young adults	M	PA, SA, EA, PN, EN	Pyrosequencing	IL6 promoter DNAm↓	Pro-inflammatory response to stress	Janusek et al., 2017
MDD patients	72% F	PA, SA, EA, PN, EN	Microarray	BDNF promoter DNAm↑	BDNF polymorphisms collaborated with ELS	Ferrer et al., 2019

MDD, major depression disorder; F, female; M, male; EA, emotion abuse; SA, sexual abuse; PA, physical abuse; EN, emotion neglect; PN, physical neglect; DNAm, DNA methylation; NR3C1, neuron-specific glucocorticoid receptor; SLC6A4, serotonin transporter; BDNF, brain-derived neurotrophic factor.

epigenetic changes. Another possibility is genetic preconditions that may depict the epigenetic profiles resistant or vulnerable to ELS. Therefore, we summarized the research into ELS in **Table 1**. We emphasized the demographic features and measured methods of epigenetic profiles. Even in studies that supported that ELS was associated with the epigenetic alterations of the disturbed HPA axis, serotonin, and neurogenesis, debates remain as to whether epigenetic changes resulted from ELS or whether

those changes were significant enough to promote the risk for depression (Wankerl et al., 2014; Alexander et al., 2018; Fiacco et al., 2019).

EPIGENETIC STUDIES FOCUSED ON ANIMALS

In addition to clinical research, preclinical studies were also conducted in pursuing the role of ELS-mediated epigenetic profiles in depression. As for the preclinical investigations, rodents were the most used materials. Of behaviors of ELS, maternal deprivation, improper maternal care, and other maltreatments were found to affect the epigenetic profiles of the animals. With regard to the animal models who developed depression, ELS enhanced the effect of adversities and made the models susceptible to depressive symptoms (Zhang et al., 2013, 2015). It was reported that chronic and unpredictable maternal separation (MS) altered the status of DNAm in the promoter of several candidate genes in the germline of the separated males, and the epigenetic characteristics possibly transmitted across generations (Franklin et al., 2010).

Similarly, the epigenetic modifications that occurred in animals were associated with the regulation of the HPA axis, monoamine, and neurogenesis. Firstly, the promoters of glucocorticoid receptors were differently methylated and these alterations were correlated with the heterogeneous ELS and evaluated timing. It was reported that maternal licking and grooming and arched-back nursing negatively correlated with the DNAm level, while being positively associated with the histone acetylation status of the GR promoters or the CpG island shore in the hippocampus (Bockmühl et al., 2015). Both the DNAm and histone acetylation jointly modulated the activity of the glucocorticoid receptor. Moreover, these epigenetic changes persisted into adulthood and can be reversible by a histone deacetylase regulator or methyl supplementation. Specifically, central infusion of a histone deacetylase inhibitor or methyl supplementation removed the difference of DNAm, histone methylation, GR expression, and HPA axis to stress between high maternal and low maternal care (Weaver et al., 2004, 2005). However, the epigenetic alterations may be sex-specific and strain-dependent, because Kundakovic et al. (2013) showed that maternal separation increased hippocampal *Nr3c1* DNAm in C57BL/6J males only, other than in Balb/cJ mouse strains. Furthermore, in C57BL/6J females, decreased hippocampal *BDNF* expression bonded to MS, while MS-induced the increased hippocampal BDNF level in male and female Balb/cJ offspring. Therefore, in confronting ELS, different animal-strains and responded elements showed discrepancies (Kundakovic et al., 2013). It is worth mentioning that the epigenetic changes of inconsistent strains that responded to ELS was also illustrated in a study investigating histone modification. In the model of MS, mice obtained a decreased expression of mRNA encoding the histone deacetylases (HDACs) 1, 3, 7, 8, and 10 in the forebrain neocortex which was followed by an increased expression of acetylated histone H4 proteins. This epigenetic modification was only observed in the Balb/c strain other than the more resilient C57Bl/6 strain. However, the upregulation of the histone deacetylase (HDAC) and histone hypoacetylation level was in an ELS model of MS tested on Sprague–Dawley rats in the ventral tegmental area. While an HDAC inhibitor was able to reverse the histone hypoacetylation and normalize the BDNF level (Shepard et al., 2018). The histone modifications likely occurred as compensation because the reversal of the histone modifications worsened the abnormal emotional symptoms resulting from ELS and interacted with antidepressants (Levine et al., 2012). Back to the conversation of the HPA axis, the corticotrophin-releasing hormone (CRH) is the upper regulator for the cortisol stress response. One study showed that MS was associated with the decreased methylated *crh* promoter and enhanced *crh* transcriptional responses to stress in adulthood (Chen et al., 2012). Moreover, increased histone H3 acetylation was also observed in MS rats, and an enriched environment reversed the epigenetic alterations and alleviated the upgraded CRH level and some phenotypes (Wang et al., 2014). Additionally, arginine vasopressin (AVP), expressed in the hypothalamic paraventricular nucleus, may coordinate with CRH in modulating the release of corticosteroids and adjusting the HPA axis in stress coping. Murgatroyd et al. (2009) showed that ELS led to hypomethylation of a key regulatory region of the Avp gene and was accompanied with consistent Avp expression in the hypothalamus. Moreover, proopiomelanocortin (Pomc), encoding POMC protein which serves as a prohormone for ACTH, was upregulated because of decreased DNAm of the critical region of the Pomc gene. The epigenetic alteration occurred soon after MS and persisted for a long time (Wu et al., 2014). However, another study assumed that the turbulent function of the HPA axis was not attributed to the DNAm status of the exon 1_7 GR promoter region when the epigenetic profile was measured 7 days after maternal separation in Sprague Dawley rat pups (Daniels et al., 2009). This difference may result from strains and timeline discrepancy. Kosten and Nielsen (2014) found that litter and sex influenced the value of maternal pup licking and licking difference attributed to the discrepancy of DNAm of Nr3c1 exon 1_7 promoters in the hippocampus and cerebellum in 35-day postnatal Sprague-Dawley rats. Therefore, though a few inconsistent outcomes were possibly derived from heterogeneous ELS model and evaluated timing, ELS was assumed to disturb the normal function of HPA axis via increased DNAm or inhibiting the transcription of GRs (Weaver et al., 2004; Daniels et al., 2009; Kosten and Nielsen, 2014). Furthermore, CRH, AVP, and POMC may collaborate to regulate the release of glucocorticoids (Murgatroyd et al., 2009; Chen et al., 2012; Wang et al., 2014; Wu et al., 2014).

In addition to the theory that ELS-induced epigenetic alterations enhanced the risk for mental sickness, Kinnally et al. (2011) proposed that an increased 5-methylcytosine (5mC) level contributed to the effect of ELS. In this female bonnet macaque study, DNAm did not differ based on early life stress. Although, increased 5-HTT and whole-genome 5mC levels reacted sharply to the ELS (Kinnally et al., 2011). Basically, 5-Hydroxymethylcytosine (5hmC) regulates DNA demethylation, while 5mC mediates DNAm. Adult female mice exposed to ELS and who had anxiety-like behaviors develop

TABLE 2 | Early life stress modulates epigenetic profiles in non-human animals.

Animal strains	Types of ELS	Measured methods	Epigenetic outcome	Phenotypic bridge	References
Long-Evans hooded rats	Decreased maternal care	Sodium bisulfite mapping + Chromatin immunoprecipitation	GR promoter DNAm↑ and histone acetylation↓	Impaired HPA axis to stress and can be reversed by the environment or medication	Weaver et al., 2004, 2005
Sprague Dawley rats	MS/MD	Site-specific accumulation and sequencing	No GR promoter DNAm occurred at 21 days postnatal	Dysfunctional HPA axis not associated with GR epigenetic profile	Daniels et al., 2009
Sprague Dawley rats	MS/MD	Pyrosequencing	CpGs of CRH promoter DNAm↑	HPA axis hypersensitivity	Chen et al., 2012
Sprague Dawley rats	MS/MD	Immunoprecipitation	Histone H3 acetylation↑ and cytosine methylation↓ in Crh promoter region	Hippocampal synaptic dysfunction and memory defects	Wang et al., 2014
C57Bl6/J **mice**	MS/MD	Pyrosequencing	Altered germline DNAm profile in males only	Male offspring easily obtained epigenetic profiles	Franklin et al., 2010
Female bonnet macaques	Programmed adversities	Pyrosequencing + Whole-genome methylation ELISA	Whole-genome 5mC↑	Aberrant stress reactivity	Kinnally et al., 2011
C57BL/6J and Balb/cJ **mice**	MS/MD	Pyrosequencing	BDNF DNAm↑ in Balb/c mice and Nr3c1 DNAm↑ in C57BL/6J males	The discrepancy between the epigenetic profile and gene expression. Sex and strains differed epigenetically	Kundakovic et al., 2013
Sprague Dawley rats	MS/MD	Immunoprecipitation	Histone H3 acetylation↓ and DNMT1 and DNMT3a↑	Decreased BDNF protein	Park et al., 2018
Wistar rats	Early-life stressful social experience	Quantitative real-time PCR + Western blot	Dnmt3a↑, H3K14ac↑, and H3K9ac↓	Decreased BDNF protein	Karen and Rajan, 2019
Sprague Dawley rats	MS/MD	Quantitative real-time PCR + Chromatin immunoprecipitation	Acetylated histone H3 and H4 at BDNF promoter IV↓	Decreased BDNF protein	Seo et al., 2016
Balb/cJ and C57Bl/6J mice	MS/MD	Real-time RT-PCR + Western blot	Histone deacetylases↓ and acetylated histone H4 proteins↑ in Balb/c mice but not C57Bl/6mice	Medications helped to modulate epigenetic profiles/alleviated depressive symptoms	Levine et al., 2012
Sprague Dawley **rats**	Decreased maternal care	Pyrosequencing	GR promoter DNAm↑ in hippocampus and cerebellum	Litter and sex differed in epigenetic profiles	Kosten and Nielsen, 2014
Long-Evans outbred rats	Caregiver maltreatment	Global DNA and Locus-specific detection	5-mC↑ in males' hippocampus, 5-hmC↓ in males' amygdala, BDNF DNA↑ in males' and females' amygdala and hippocampus	Sex-specific and region-specific DNAm profiles in the whole genome	(Doherty et al., 2016)
C57BL/6J mice	Early-life environmental stress	Genome-wide sequencing	Altered 5hmC level	Depressive symptoms	Papale et al., 2017
C57BL/6 mice	MS/MD	Immunoprecipitation + ChIP-seq	Altered genomic landscape of H3K4me3	Reduced locomotor activity and reduced exploratory activity	Ershov et al., 2018
Sprague Dawley rats	MS/MD	Real-time reverse transcription PCR + Western blot	miRNA-504↑	Decreased dopamine receptor D2 expression	Zhang et al., 2013
Sprague Dawley rats	MS/MD	Real-Time reverse transcription quantitative PCR + Western blot	miRNA-9↓ and miRNA-326↑ in the striatum	Increased dopamine receptor D2 expression	Zhang et al., 2015

MS, maternal separation; MD, maternal deprivation; GR, glucocorticoid receptor; 5-mC, 5-methylcytosine; 5-hmC, 5-hydroxymethylcytosine; DNMT, DNA methyltransferase; BDNF, brain-derived neurotrophic factor.

found disruptions of hypothalamic 5hmC and possibly an expression of the stress-related genes (Papale et al., 2017). Furthermore, male rats exposed to caregiver maltreatment had higher 5mC levels in the hippocampus and had lower 5hmC levels in the amygdala (Doherty et al., 2016). Moreover, early-life stressful social experiences elevated the level of DNA methyltransferases (Dnmt3a), ten-eleven translocation (Tet3), methyl-CpG-binding protein-2 (MeCP2), and repressor element-1 silencing transcription factor (REST) in the amygdala of adolescents and adults. The alterations of these DNAm regulators were assumed to modulate the level of DNAm and collaborated with the histone modifications that regulate phenotypes in response to ELS and current stressful events (Karen and Rajan, 2019). Therefore, ELS may alter epigenetic

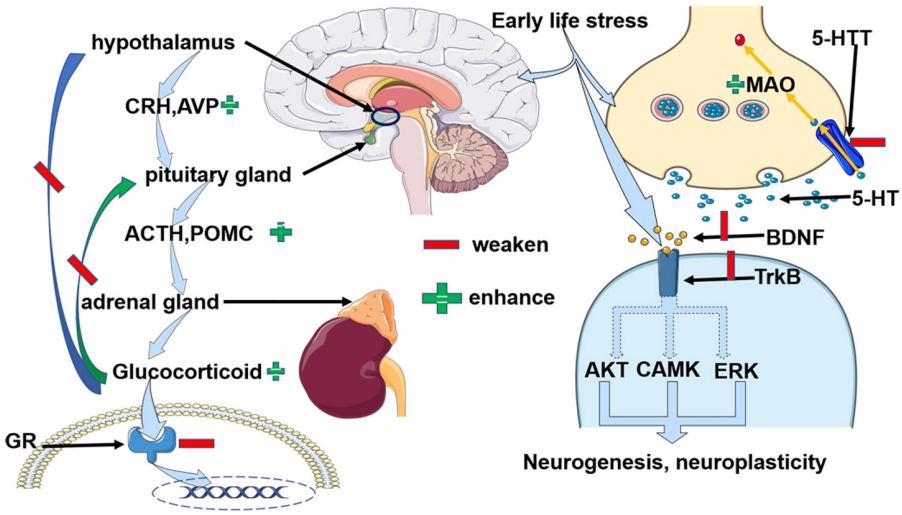

FIGURE 1 | Early life stress-induced alterations of three depressive-related paths, the HPA axis, 5-HT, and BNDF, respectively due to epigenetic modification. 1) Early life stress impaired the GRs and paralleled with the dysfunctional negative feedback loop of the HPA axis. Specifically, the enhanced expression of CRH and ACTH, and abundant glucocorticoid was observed. Moreover, AVP, POMC, and FKBP5 KITLG collaborated to regulate the function of HPA axis.2) Early life stress primarily weakened the activity of 5-HTT but enhanced MAO activity. Therefore, early life stress suppressed the recycling of 5-HT and facilitated its degradation. 3) Early life stress inhibited the BDNF and TrkB and indirectly impaired the neurogenesis and neuroplasticity via AKT, CAMK, and ERK pathways. Abbreviation: CRH: corticotrophin-releasing hormone; AVP: arginine vasopressin, ACTH: adrenocorticotropic hormone; POMC: proopiomelanocortin, GR: glucocorticoid receptor; 5-HT: 5-hydroxytryptamine; 5-HTT: serotonin transporter; BDNF: brain-derived neurotrophic factor; TrkB: tropomyosin receptor kinase B; CAMK: calmodulin-dependent kinase, FKBP5: FK506 binding protein 5, KITLG: Kit ligand gene.

profiles linked to gain risk for depression via gene manipulation or global regulators variation.

As for the molecules involved in depression, BDNF is the most well-known. Roth et al. demonstrated that rats exposed to predominately abusive behaviors produced persistent DNAm of *BDNF* in the adult prefrontal cortex and the epigenetic changes transmitted into the next generation (Roth et al., 2009). Similarly, the altered profile of DNAm induced by MS or other adversities was observed in the germline of C57Bl6/J males and Balb/c mice and can be transmitted through generations (Franklin et al., 2010; Kundakovic et al., 2013, 2015; Park et al., 2018; Shepard et al., 2018). Moreover, ELS contributed to decreased histone H3 acetylation levels binding to the BDNF exon I promoter and increased DNMT1 and DNMT3a mRNA levels in the hippocampus (Park et al., 2018). Additionally, maternal deprivation and current stress induced different contents of depressive symptoms in Sprague-Dawley rats. However, it was maternal deprivation but not current stress rats that contributed to downregulated hippocampal BDNF and higher miR-16 expression (Bai et al., 2012). Moreover, female rats exposed to caregiver maltreatment had greater BDNF DNAm in the amygdala and hippocampus (Doherty et al., 2016). With regard to the histone modifications, Seo and colleagues proposed that ELS built the background for the sensitivity of stress via decreased levels of acetylated histone H3 and H4 at BDNF promoter IV and restraint stress enhanced the epigenetic changes and thereafter deteriorated the depressive phenotypes (Seo et al., 2016). Therefore, types of ELS attributed to the epigenetic inhibition of BDNF and decreased the activity of BDNF in modulating neuroplasticity and neurogenesis.

In addition to the neuroplasticity specifically involved in the derived neurotrophic factor, glutamate receptor, and histone modulated regulators were also investigated. The type I metabotropic glutamate receptor (mGluR1) regulated the synaptic plasticity and *Grm1* encodes this protein. Bagot et al. (2012) found that higher pup licking/grooming rats were associated with decreased DNAm and increased levels of histone 3 lysine 9 acetylation and histone 3 lysine 4 trimethylation of *Grm1* in the hippocampus. The histone modification was negatively associated with the DNAm and positively correlated with *Grm1* transcription and translation. Therefore, good maternal care ensured well-operated receptor function (Bagot et al., 2012). Besides, Wang et al. demonstrated that male Sprague-Dawley rats who suffered early-life social isolation had increased levels of neuronal H3K9me2 (a repressive marker of transcription) in the hippocampus, accompanied by decreased expression of hippocampal N-methyl-D-aspartate (NMDA) receptor subunits, and the AMPA receptor subunits, GluR1 and GluR2. Thus, the epigenetic changes disturbed or deteriorated the neural plasticity, while antidepressants that reversed the epigenetic changes restored the neuroplasticity (Wang et al., 2017). As regards to the histone modulators, dynamic changes differed. Specifically, ELS decreased the level of H3K14 acetylation (ac) and H3K9ac in adolescents and then increased it in adults. While H3K4 methylation (me2/me3) levels were elevated in adolescents and adults, and H3K9me2/me3 levels increased in adults (Karen and Rajan, 2019). Moreover, histone modifications also modulated the pro-inflammatory characteristics in rats exposed to the ELS. It was assumed that chronic unpredictable mild stress

during the adolescence period combined with maternal separation induced depressive-like behaviors, burst cytokines, and increased Jmjd3 (a histone H3 lysine 27 (H3K27) demethylase) and decreased H3K27me3 expression in the prefrontal cortex and hippocampus of both adolescent and adult rats (Wang et al., 2018, 2020). Therefore, compared with the studies of the site-specific profile of DNAm, DNAm, and histone regulators seemed to have a more profound effect. Further studies should focus on finding more targets of these modulated regulators.

With respect to the miRNAs, epigenome or regional miRNAs have been investigated. MS or chronic unpredictable mild stress increased the level of miR-16, miR-504, miR-326, miR124a, and decreased miR-9 and miR-135a in respective regions (Bai et al., 2012; Zhang et al., 2013, 2015; Bahi, 2016; Liu et al., 2017; Xu et al., 2017). Of these microRNAs, miR-16, and miR124a correlated negatively with BDNF expression in the rats' hippocampus which ELS exposed (Bai et al., 2012; Bahi, 2016), and miR-504 was negatively related with lower dopamine receptor D1 (DRD1) and D2 (DRD2) expression in the nucleus accumbens of the rats (Zhang et al., 2013). While in another study, maternal deprivation-induced DRD2 mRNA expression was accompanied by decreased miR-9 in the striatum (Zhang et al., 2015). However, although substantial alterations of miRNAs were observed in ELS models, the precise mechanisms of how and what these miRNAs transcriptions regulated are unknown. Consequently, the underlying relationship between miRNAs and depressive phenotypes needs to be clarified and how miRNA interacted with DNAm and histone modification requires more exploration.

In conclusion, according to the studies that focused on the non-human animals, ELS was associated with an increased DNAm of the glucocorticoid receptor and BDNF (Weaver et al., 2004; Kosten and Nielsen, 2014; Doherty et al., 2016; Park et al., 2018). While genes contributing to glucocorticoid release were upregulated. Additionally, alterations of histone modifications binding to specific regions or genome-wide and miRNAs also led to decreased GR and BDNF expression, while permitted the CRH, AVP, and POMC expression (Murgatroyd et al., 2009; Chen et al., 2012; Wang et al., 2014; Wu et al., 2014). The epigenetic modifications reacting to ELS were listed in **Table 2**. These changes were assumed to result in the aberrant HPA axis and disrupt neurogenesis and neuroplasticity. Moreover, compared with the epigenetic investigations focused on the human subjects, the non-human studies showed more consistent outcomes when strains and ELS interventions remained the same (Kundakovic et al., 2013; Kosten and Nielsen, 2014).

CONCLUSION AND DISCUSSION

ELS is prevalent among humans, and the relationship among ELS, the dynamic epigenetic alterations, and depression have been investigated in recent years. Epidemiological studies encouraged that ELS could possibly increase the risk of depression and enhance the adverse effect of later life stress. However, different types of early adversities and sex had considerable effects on the consequences (Kim et al., 2013; Culpin et al., 2015; St Clair et al., 2015; Bjorkenstam et al., 2017; Dahl et al., 2017; Frodl et al., 2017; Opel et al., 2019). The epigenetic alterations may help to explain how ELS modulated the epigenetic reprogramming and what the effect of these alterations was. Therefore, we cited the evidence that displayed the relationship, and most studies agreed that epigenetic changes were observed in humans and animal models following ELS (Weaver et al., 2004; McGowan et al., 2009; Chen et al., 2012; Kang et al., 2013; Ouellet-Morin et al., 2013; Bockmühl et al., 2015; Bustamante et al., 2016; Montirosso et al., 2016; Tyrka et al., 2016; Palma-Gudiel et al., 2018; Peng et al., 2018). Compared with individuals free of ELS, early life adversities were associated with substantial epigenetic alterations. The epigenetic drifts modulated the function of the HPA axis, monoamine, and neurogenesis and neuroplasticity (Weaver et al., 2004; McGowan et al., 2009; Perroud et al., 2011; Chen et al., 2012; Wang et al., 2014; Bockmühl et al., 2015; Tyrka et al., 2016; Palma-Gudiel et al., 2018; Peng et al., 2018). As for the studies, they mostly supported that ELS increased the risk of depression and that epigenetic reprogramming contributed to the dysfunctional response to stress. Specifically, at least altered DNAm and histone activity helped to deactivate GR and destroy the negative feedback regulation of the HPA axis. The abnormal HPA axis function decreased the threshold for developing depression.

In addition to the regulation of the HPA axis, dysregulated monoamine in the brain or peripheral blood resulting from epigenetic adjustments were also observed. The alternative epigenetic changes of 5-HT were illustrated. Increased DNAm or histone modifications of 5-HTT related genes were associated with ELS (Kinnally et al., 2011; Kang et al., 2013; Melas et al., 2013; Wankerl et al., 2014; Montirosso et al., 2016; Fumagalli et al., 2018). While exhausted 5-HT in the synapse and the blunt cortisol response enlarged the adverse event in later life (Ouellet-Morin et al., 2013). Therefore, it was likely epigenetic regulations that modulated the background for depression development and deterioration in the context of ELS.

Furthermore, molecules involved in the neurogenesis and neuroplasticity were also reshaped corresponding to the ELS. According to most of the evidence, increased DNAm and altered histone modifications of the genes promoted neurogenesis and neuroplasticity which was also observed in individuals or animals with ELS (Roth et al., 2009; Bai et al., 2012; Doherty et al., 2016; Seo et al., 2016; Ferrer et al., 2019). The imbalanced epigenetic modulations bestowed susceptibility to depressive symptoms.

The profile of miRNAs also changed when confronted with ELS and played a role in promoting depression. Depression and ELS bilaterally contributed to the dynamic turbulence of miR-16, miR-504, miR-326, miR124a, miR-9, and miR-135a. However, though part of the network of miRNAs was illustrated, how these collaborated remains unknown. Furthermore, what the targets are of these miRNAs stayed largely undisclosed (Bai et al., 2012; Zhang et al., 2013, 2015; Bahi, 2016; Liu et al., 2017; Xu et al., 2017). Of note, broad research should analyze the network for these miRNAs.

As regards the epigenome-wide study, a number of epigenetic adaptations were discovered. Meanwhile, because of the large amounts of influenced genes discovered and dynamic epigenetic regulations across life, an assortment of biological activities were involved (Franklin et al., 2010; Kinnally et al., 2011; Doherty et al., 2016; Papale et al., 2017; Wikenius et al., 2019). However, it is difficult to qualitatively and quantitatively measure the role of these changes induced by ELS and how much they accounted for the onset of depression.

Apart from the research that supported the role of early life stress on the epigenetic profiles in depression, few researchers assumed that no significant evidence of epigenetic changes following ELS increased the risk for depression or that ELS was not responsible for depression formation based on epigenetic proof (Daniels et al., 2009; Kundakovic et al., 2013; Bustamante et al., 2016; Alexander et al., 2018; Fiacco et al., 2019). One theory of depression requires two-hit or multiple-hit stressful events. The development of depression requires early adversities and the following burden strengthens ill-programmed epigenetic profiles. In the individuals who were exposed to ELS but remained healthy in adults, the epigenetic profiles differed compared with the individuals who later developed depression. Furthermore, genetic background and risk of polymorphisms of genes were more likely to obtain epigenetic reprogramming tilted toward depression (Peña et al., 2013; McCoy et al., 2016; Alexander et al., 2018; Ferrer et al., 2019). Additionally, the contents and severity of ELS was associated with different epigenetic profiles. In human, physical abuse, sexual abuse, emotional abuse, and physical & emotional neglects were the most studied (Kim et al., 2013; Culpin et al., 2015; St Clair et al., 2015; Airagnes et al., 2016; Opel et al., 2019). While in non-human research, maternal deprivation/separation and prenatal stress were greatly explored. Besides, especially for the human beings, consistent stress continuously reprogramed the epigenetic profiles (Weaver et al., 2004; Murgatroyd et al., 2009; Roth et al., 2009; Zhang et al., 2013; Seo et al., 2016; Reus et al., 2017; Xu et al., 2017). Additionally, ELS was mostly measured retrospectively in humans and commonly individuals suffered from more than one adversity (St Clair et al., 2015; Williams et al., 2016; Bjorkenstam et al., 2017; Opel et al., 2019). This situation likely contributed to heterogeneous outcomes when epigenetic characteristics were measured. Furthermore, with regard to the studies of non-human animals, though the models of ELS were well-controlled, the strain and measured timing possibly led to incongruous results (Weaver et al., 2004; Murgatroyd et al., 2009; Chen et al., 2012; Levine et al., 2012; Seo et al., 2016). Moreover, sex differences influenced the development of depression due to ELS in both human and non-human animals (Kundakovic et al., 2013; Melas et al., 2013; Kosten and Nielsen, 2014; St Clair et al., 2015; Frodl et al., 2017). Although compared with humans, the epigenetic profiles correlated with ELS of non-human animals were relatively consistent when sex, strain, and measured methods remained the same (Weaver et al., 2004, 2005; Murgatroyd et al., 2009; Wang et al., 2014).

In conclusion, ELS might increase the risk of depression by building a susceptible background for the depression (Kim et al., 2013; Bjorkenstam et al., 2017; Dahl et al., 2017; Opel et al., 2019). Epigenetic alterations may add fuel to the fire in the development of depression when individuals possess a history of early life stress and a risk genetic property. We concluded that the possible epigenetic outcomes of ELS induced changes that shared common biological changes with depression as in **Figure 1**. Due to the fact that the epigenetic changes suppressed the expression of GR, and elevated CRH, AVP, and ACTH expression, the HPA axis was dysfunctional. Specifically, negative feedback was destroyed and continuously higher glucocorticoids circulated within the body. An excessive amount of glucocorticoids affected inflammatory and neuroplastic activities. On the other hand, ELS decreased the expression of 5-HTT and inhibited the transportation of 5-HT to the presynaptic neurons. In the meantime, enhanced monoamine oxidase (MAO) degraded the 5-HT. Moreover, in the subsynaptic membrane, decreased BDNF expression selectively resulted in knockdown of the TrkB receptor which led to the adverse effect on the neurogenesis and neuroplasticity (Weaver et al., 2004, 2005; McGowan et al., 2009; Chen et al., 2012; Kang et al., 2013; Ouellet-Morin et al., 2013; Tyrka et al., 2016; Peng et al., 2018; Prowse et al., 2020). More research needs to be done to investigate the direct evidence for the role of early life stress-induced epigenetic changes when contributing to the vulnerability of depression because of the uncertainty proposed by finite evidence. Furthermore, scientists should move on to explore the dynamic epigenetic adaptations when individuals confronted ELS and later depression. It is possible that a group of people obtained ELS when the epigenetic program was active, but remained healthy in later life, epigenetic alterations could be an adaptive evolution. Therefore, exploring the regulators to induce the epigenetic changes to accommodate stress events will be promising.

AUTHOR CONTRIBUTIONS

ML wrote the first draft. XF, WX, WG, BL, RC, and WY made major revisions to the logic of this article. WY provided critical revisions. All authors approved the final version of the manuscript for submission.

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Early-Life Stress Alters Synaptic Plasticity and mTOR Signaling: Correlation With Anxiety-Like and Cognition-Related Behavior

Anfeng Wang[1], Xiaojuan Zou[1], Jiajia Wu[1], Qingyu Ma[2], Naijun Yuan[2], Fengmin Ding[1], Xiaojuan Li[2] and Jiaxu Chen[1,2,3]**

[1] School of Basic Medical Science, Hubei University of Chinese Medicine, Wuhan, China, [2] Formula-Pattern Research Center, School of Traditional Chinese Medicine, Jinan University, Guangzhou, China, [3] School of Traditional Chinese Medicine, Beijing University of Chinese Medicine, Beijing, China

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***Correspondence:**
Xiaojuan Li
lixiaojuan@jnu.edu.cn
Jiaxu Chen
chenjiaxu@hotmail.com

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Early-life stress (ELS) predisposes individuals to psychiatric disorders, including anxiety and depression, and cognitive impairments later in life. However, the underlying molecular mechanisms are not completely understood. Developmental deficits in hippocampal synaptic plasticity are among the primary detrimental alterations in brain function induced by ELS. Impaired synaptic plasticity is usually accompanied by decreased synaptic proteins, such as postsynaptic density 95 (PSD95) and synaptophysin, which are important for synaptic function. The mTOR signaling pathway plays a vital role in regulating protein translation, and mTOR activation is functionally associated with synaptic protein synthesis. In the present study, we observed whether ELS impacts synaptic protein synthesis and mTOR signaling, which is involved in synaptic plasticity. Herein, we established a maternal separation (MS) and chronic restraint stress (CRS) model and evaluated anxiety-like behavior and cognitive function (e.g., learning and memory) in adulthood through behavioral examination and analyzed hippocampal expression levels of PSD95 and synaptophysin. To explore whether the mTOR signaling pathway was associated with ELS, we also examined the activity of mTOR and s6. The behavior tests indicated that maternally separated mice showed increased anxiety-like behavior and cognitive impairments. PSD95 and synaptophysin mRNA and protein expression levels were decreased in the hippocampus, and phosphorylated mTOR and phosphorylated s6 were significantly decreased in maternally separated mice vs. those not exposed to MS. Our data demonstrate that MS impairs synaptic plasticity and inhibits mTOR signaling, specifically via s6. Therefore, we speculate that ELS decreased synaptic plasticity via the inhibition of the mTOR pathway in the hippocampus, which may underlie vulnerability to stress and mental disorders in adulthood.

Keywords: early-life stress, maternal separation, synaptic plasticity, mTOR, s6

INTRODUCTION

Early-life stress (ELS), such as experiencing emotional neglect, physical abuse or traumatic events, can lead to long-lasting changes in neuronal physiology (Fumagalli et al., 2007). Clinical and epidemiological studies have suggested that individuals exposed to early adverse experiences have an increased risk of mental disorders, including anxiety and depression, and cognitive deficits (Harkness et al., 2006; Carr et al., 2013; Ménard et al., 2016). Maternal separation (MS) is an animal model of ELS that has been widely used in recent decades (Vetulani, 2013; Kambali et al., 2019). Several studies have reported that MS negatively impacts brain function, resulting in increased anxiety- and depressive-like behaviors and impaired cognitive function (Boccia et al., 2007; Desbonnet et al., 2010; Nishi et al., 2014). Thus, it has been suggested that adverse experiences in early life may induce vulnerability to the effects of stress later in life. However, the mechanisms by which this occurs are still not completely understood.

The regulation of synaptic plasticity is closely related to the induction of mental disorders. Based on existing evidence, ELS affects synaptic function and impairs synaptic plasticity (Sousa et al., 2014; Jeanneteau and Arango-Lievano, 2016). It has been previously reported that decreased postsynaptic density 95 and synaptophysin levels were found in MS rats (Martisova et al., 2013; Dandi et al., 2018). Mammalian target of rapamycin (mTOR) is a protein kinase that belongs to the phosphatidylinositol 3-kinase-related kinase protein family, which integrates signals from neuronal activity, growth factors, and nutrient levels to regulate the initiation of protein translation (Abelaira et al., 2014). Interestingly, it has been reported that mTOR is involved in translation control and long-lasting synaptic plasticity (Hoeffer and Klann, 2010). Previous studies have shown that reduced mTOR signaling function could result in decreased synthesis of synaptic proteins (Duman et al., 2016). Dysregulation of mTOR can lead to various mental illnesses (Hoeffer and Klann, 2010). Clinical studies have also found deficits in the mTOR signaling pathway in subjects with major depressive disorder (Jernigan et al., 2011), and activation of the mTOR pathway is related to antidepressant actions (Suo et al., 2013). However, it is poorly understood whether ELS affects mTOR signaling.

In the present study, we evaluated the effects of MS and subsequent chronic restraint stress (CRS) on behavior and synaptic proteins in the hippocampus of mice. In addition, we investigated whether the changes in behavior and synaptic plasticity were accompanied by inhibition of the mTOR pathway in the hippocampus.

MATERIALS AND METHODS

Animals

All protocols involving experimental animals were reviewed and approved by the Institutional Animal Care and Use Committee at Jinan University (approval No. IACUC-20190702-03). C57BL/6J female mice that were 15 days pregnant were purchased from the Experimental Animal Center at Guangzhou University of Chinese Medicine and individually housed until delivery. The day of delivery was considered postnatal day zero (postnatal day 0; PND0). Male pups were used in the present study. All animals were housed in a room at a temperature of 22 ± 2°C and 40–45% humidity and on a 12-h light/dark cycle and they were allowed free access to chow and water. Behavioral testing began at PND60. At PND69, all mice were euthanized, and hippocampal tissues were separated on 4°C ice for real-time PCR and western blotting experiments.

Experimental Design

The male pups were assigned to three groups of 9–10 mice per group. The control group consisted of non-separated, non-restrained mice. The CRS group consisted of non-separated, restrained mice. The MS + CRS group consisted of maternally separated and restrained mice (**Figure 1** for the experimental design).

Maternal Separation and Chronic Restraint Stress Procedure

The MS procedure was initiated at PND1, and the protocol was performed as previously described (McCoy et al., 2016; Park et al., 2018), with slight modifications. From PND1 to PND21, the pups were removed from home cages to separate them from their dams and placed in another separate room for 4 h (8:00–12:00) a day. Following a 4-h separation period, the pups were then returned to their original cages and cared for by their mothers. Non-separated pups stayed with their dams and remained undisturbed in their cages (except for cage cleaning once every 3 days) until PND22.All litters were weaned and separated by sex at PND22, and only male pups were used in the current study.

At PND39,the pups in the CRS and MS + CRS groups were subjected to CRS according to a previously reported protocol (Campos et al., 2013; Lee et al., 2019). Briefly, the mice were placed in 50-mL conical tubes with air holes drilled into the sides to restrict their movements for 3 h (9:00–12:00 h) daily for 3 weeks.

Behavioral Testing

To detect changes in behavior and cognition (e.g., learning and memory), mice were subjected to behavioral tests that were performed the day after CRS ended (**Figure 1** for timeline). The mice were placed in the test room to adapt to the environment for more than 1 h before each behavioral test. Ethovision (v.14.0, Noldus Information Technology) was used to record and analyze mouse behavior.

Open Field Test

Anxiety-like behavior and locomotor activity were assessed in the open field test (OFT) (Lau et al., 2008). The open-field apparatus used in this study was a square arena (50 cm× 50 cm) surrounded by 40 cm high black walls. The arena's white floor was divided into 25 equal squares by black lines. During the test, the mice were placed at the center of the arena. In this test, the mice were individually placed at the center of the arena and allowed to freely explore the apparatus for 5 min. Between tests, the apparatus was cleaned with 70% ethanol to prevent the smell of the previous

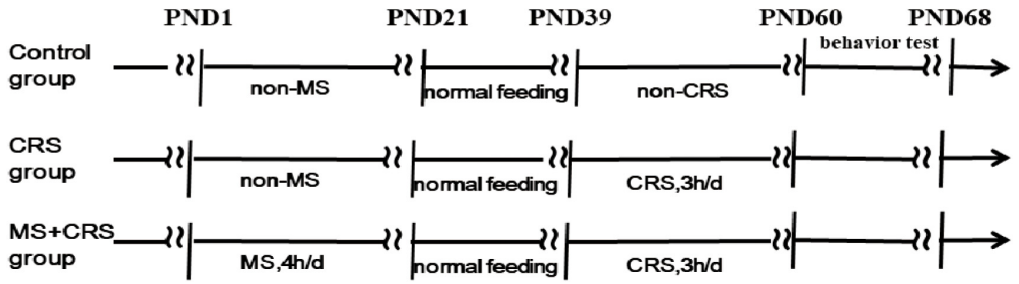

FIGURE 1 | Schematic representation of the experimental design.

mouse influencing the subsequently tested mice. The time spent in the center area and total distance moved were evaluated.

Elevated Plus Maze

The elevated plus maze (EPM) test was performed to measure anxiety-like behavior in the current study. The maze consisted of two open arms (67 cm × 7 cm) and two enclosed arms (67 cm × 7 cm × 14 cm), which were placed 65 cm above the floor. Each mouse was placed in the central zone of the maze and left to freely explore for 5 min. Decreased time spent in and number of entries into the open arms suggest higher levels of anxiety (Walf and Frye, 2007; Dandi et al., 2018). Seventy percent ethanol was used to clean the maze as in the OFT. We analyzed the number of entries into the open arms and the ratio of open arm time (open arms time/total arms time).

Novel Object Recognition Task

The novel object recognition task (NORT) was performed to investigate non-spatial learning and memory and was carried out according to the procedure described in previous studies (Kruk-Slomka et al., 2014; Lueptow, 2017). Specifically, the test was performed in an open-field apparatus (50 × 50 × 40 cm^3) and consisted of three trials:habituation trial, training trial and testing trial. In the habituation trial, the mouse was placed into the open field without objects and allowed to explore for 5 min. After 24 h, training and testing trials were performed. First, two identical objects (two cylindrical boxes) were placed at the left and right back corners of the apparatus. Then, the mice were placed into the apparatus and allowed to explore for 10 min for the training trial. After an hour, one cylindrical box was replaced with a cube box for the testing trial, and the mice were allowed to freely explore for 10 min. Seventy percent ethanol was used to remove the olfactory cues of the object. Because animals are naturally fond of novelty, if the mouse remembered the familiar objects, it will spend more time with the novel object. Therefore, decreased time spent with the novel object suggested cognitive impairment. The exploration time of the two objects was recorded in the testing trial. The recognition index (RI) was calculated as 100% × time with the novel object/time with both objects.

Morris Water Maze

In the present study, the Morris water maze (MWM) was used to assess spatial learning and long-term memory with spatial learning and probe trials. The test was carried out according to the method described by Weitzner et al. (2015) with some modifications and conducted in 5-day blocks. The maze was a circular open pool (approximately 1.2 m in diameter) and divided into four quadrants: northeast (NE), northwest (NW), southeast (SE), and southwest (SW). The escape platform was placed in the center of the NW quadrant and submerged 0.8 cm below the water surface. Four pictures of different shapes were pasted on the walls of the four quadrants as maze cues. The acquisition trials lasted for 4 days. Each mouse received four trials per day and were released at four different positions in a semirandomized way. On each trial, the mice were placed in the water facing the pool wall and allowed to swim until locating the escape platform or until a maximum of 60 s. Upon finding the platform, the mice remained on the platform for 15 s before removal from the pool and placement in the cage. If a mouse did not locate the platform within 60 s, it was guided to the platform and remained there for 15 s before being returned to its cage. The latency to escape (time to reach the platform) and swim speed were measured for each mouse. On the fifth day, the probe trial was administered without a platform in the pool. The mouse was released from a point in the opposite quadrant (SE) and allowed to explore for 60 s. The time spent in the target quadrant and the number of crossings over the previous position of the escape platform were recorded.

Real-Time PCR

The expression of PSD95 and synaptophysin in the hippocampus of mice was measured by RT-PCR. Total RNA was isolated from hippocampal tissues using TRIzol (Invitrogen, United States), and its concentration was detected by spectrophotometry (Eppendorf, Germany). According to the instructions of the Revertaid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA), the total RNA concentrations were normalized to 20 μl before reverse transcription into cDNA. Sequences for the primers are shown in **Table 1** and were designed by Sangon Biotech Co., Ltd. (Shanghai, China). Power SYBR®Green PCR Master Mix (Thermo Fisher Scientific) was used for fluorescence qPCR to amplify samples using the following cycling parameters: 95°C for 30 min and 40 cycles of 95°C for 5 s and 60°C for 30 s. All results are expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the present study

and performed by Bio-Rad CFX Manager 1.1 (Bio-Rad, United States).

Western Blot

The expression levels of PSD95, synaptophysin, mTOR, p-mTOR, s6, and p-s6 proteins in the mouse hippocampus were tested by Western blot analysis. Total protein from hippocampal tissues was extracted in RIPA buffer with proteinase and phosphatase inhibitors. A BCA protein assay kit was used to examine the concentration of total protein, which was adjusted to 2 mg/ml. Proteins were resolved by 8 or 10% SDS-PAGE gel electrophoresis and transferred to PVDF membranes. After blocking with 5% skim milk or 90 min and then washing 2 × 10 min in TBST buffer, the membranes were incubated overnight at 4°C with primary antibody. The next day, the membranes were washed with TBST buffer and then incubated for 60 min with secondary antibody. Bands were developed by enhanced chemiluminescence reagent (Millipore, Billerica, MA, United States) and subsequently visualized by an imaging system (Bio-Rad, California, United States). Finally, Image J was used to quantify the integrated gray densities of each band. The primary and secondary antibody used in Western blotting in the present study are listed in **Table 2**.

Statistical Analysis

All data were analyzed using SPSS 20.0 (IBM software) and presented as the mean ± SEM. When the difference was at the level of $P < 0.05$, the data were accepted as statistically significant. Two-way repeated measures analysis of variance (ANOVA) was used to analyze latency to escape (time to reach the platform) and swim speed in the MWM. The remaining data were assessed using one-way ANOVA and then using *post-hoc* Tukey tests.

TABLE 1 | Primer sequences used in RT-qPCR analysis.

Gene		Primer sequence
PSD-95	Forward	5′-TCCAGTCTGTGCGAGAGGTAGC-3′
	Reverse	5′-CAGGGAGCGGGGACGGATG-3′
Synaptophysin	Forward	5′-AGTACCCATTCAGGCTGCAC-3′
	Reverse	5′-CCGAGGAGGAGTAGTCACCA-3′
GAPDH	Forward	5′-GGCAAGTTCAATGGCACAGT-3′
	Reverse	5′-AAAGTGGAGGAATGGGAGTT-3′

TABLE 2 | Primary and secondary antibody used in Western blotting.

Antigen	Host	Manufacturer	Dilution	Catalog#
PSD95	Rabbit	CST	1:1,000	3409
Synaptophysin	Rabbit	CST	1:1,000	36406
Phospho-mTOR (Ser2448)	Rabbit	Abcam	1:5,000	Ab109268
mTOR	Rabbit	CST	1:1,000	2983
Phospho-s6 (Ser240/244)	Rabbit	CST	1:1,000	5364
S6	Rabbit	CST	1:1,000	2217
Bate actin	Rabbit	Affinity	1:10,000	AF7018
Goat anti-rabbit IgG (H + L)	Goat	ZSGB-BIO	1:7,000	ZB-2010

RESULTS

Analysis of Anxiety-Like Behaviors

In this study, the OFT and EPM task, two reliable behavioral tests of anxiety, were used to evaluate emotionality. As shown in **Figures 2A,B**, in the OFT, the results showed that the CRS and MS + CRS groups were significantly different compared with the control group [$F(2, 26) = 13.55$, $P < 0.05$; $P < 0.001$] regarding the time spent in the central squares. In particular, both the CRS and MS + CRS mice spent less time in the center squares than the control mice. Moreover, the time was significantly lower in the MS + CRS group than in the CRS group ($p < 0.05$). However, the distance that the CRS and MS + CRS groups moved was not significantly different from that in the control group, which indicated that neither CRS nor MS + CRS affected the total distance moved of the mice in the OFT. **Figure 2C** shows tracking images.

Behavioral changes for the three groups observed in the EPM test are presented in **Figure 3**. As shown in **Figures 3A,B**, both the CRS and MS + CRS groups had a decreased number of entries and percentage of time spent in the open arms [$F(2, 25) = 10.611$, $P < 0.01$, $P < 0.001$; $F(2, 25) = 17.748$, $P < 0.05$, $P < 0.001$] compared with the control group. Moreover, the percentage of time spent in the open arms was lower in the MS + CRS group than in the CRS group ($P < 0.05$), but no significant difference between the above two groups was observed in the number of entries into the open arms ($P > 0.05$). These results indicated that the mice in the MS + CRS group displayed more anxiety-like behavior than those in the CRS group.

Analysis of Learning and Memory Behavior

To examine whether MS affects learning and memory, the MWM test and NORT were used in the current study to evaluate memory and spatial and non-spatial learning. As shown in **Figure 4**, the RI value in the MS + CRS mice was significantly different from that in the control mice [$F(2, 26) = 10.797$, $P < 0.001$] in the NORT. Specifically, *post-hoc* comparisons revealed that the difference in exploration time between the novel object and the familiar object was lower in the MS + CRS group than in the control group ($P < 0.05$). However, no significant difference in the RI value was observed between the CRS and control groups ($P > 0.05$). The results indicated that MS disrupted non-spatial learning and memory processes.

In the MWM, two-way repeated measures ANOVA showed that the latency to escape on the hidden platform significantly differed between group and training days [group: $F(2, 24) = 4.560$, $p < 0.05$]; training days [$F(3, 72) = 43.981$; $p < 0.001$]. As shown in **Figure 5A**, the escape latency for mice in all experimental groups was shortened with the passage of training days. On the fourth day of training, the time to reach the platform was significantly higher in the MS + CRS group than in the control group ($p < 0.05$). The latency in the CRS mice showed an increasing tendency, but the difference from the control group was not statistically significant. These

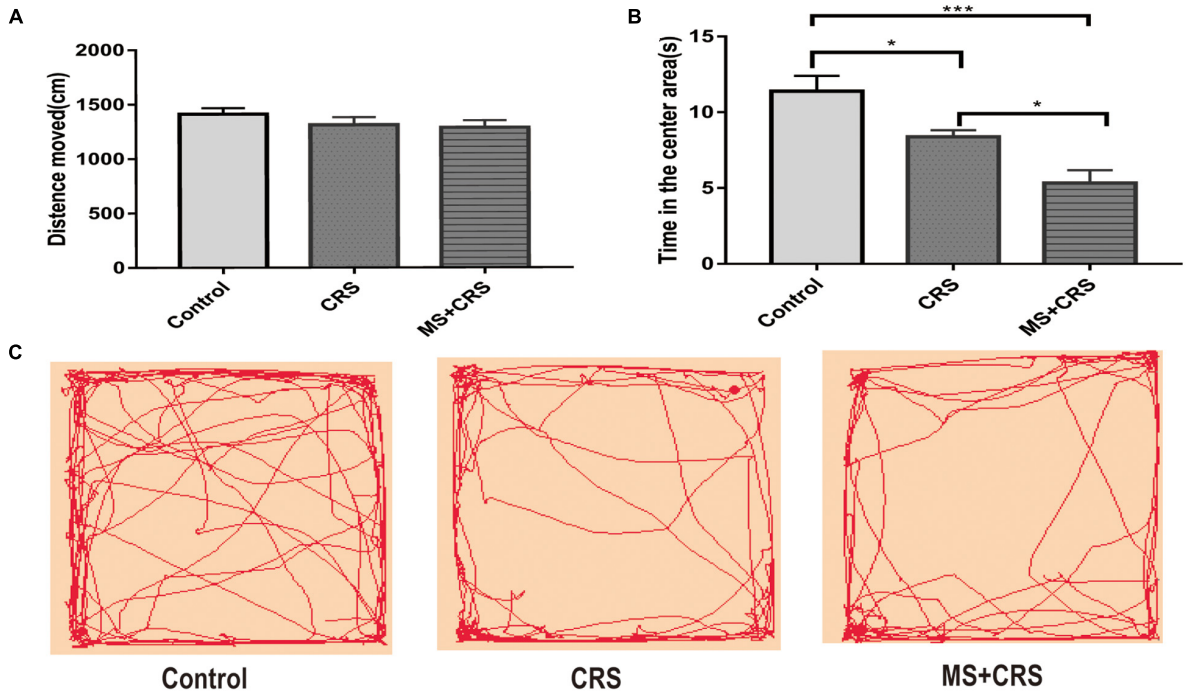

FIGURE 2 | Behavioral changes during the open field test (OFT). **(A)** Means of distance moved and **(B)** Time in the center area. **(C)** Representative video tracking images during 5 min in the OFT. Data are presented as the mean ± SEM. n = 9–10 mice in each group. $^{*}P < 0.05$, $^{***}P < 0.001$.

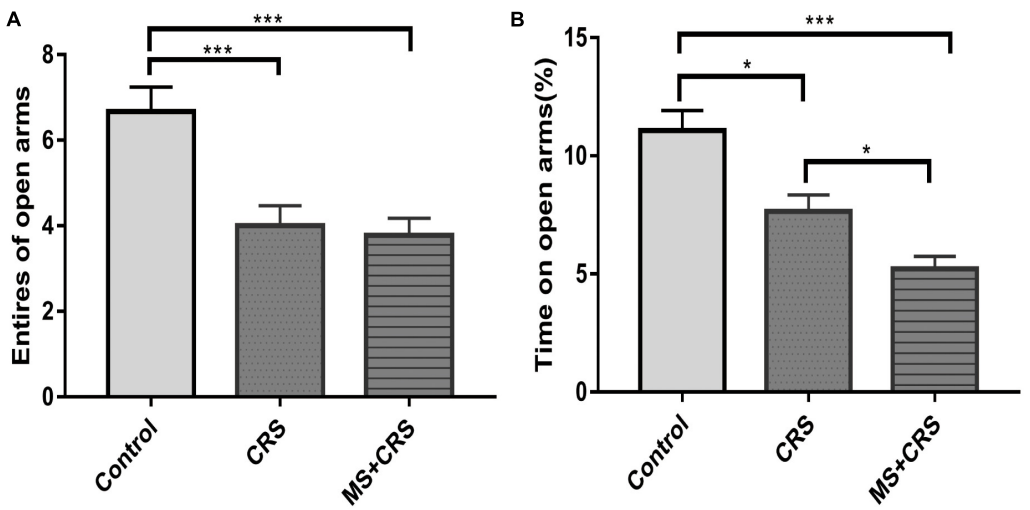

FIGURE 3 | Behavioral changes in the elevated plus maze (EPM). **(A)** Entries into open arms. **(B)** Time spent in the open arms expressed as a ratio of time spent in both open and closed arms of the EPM. Data are presented as the mean ± SEM; n = 9–10 mice in each group. $^{*}P < 0.05$, $^{***}P < 0.001$.

results indicated that spatial learning abilities were impaired in the mice in the MS + CRS group but not the CRS group. On the fifth day, a probe trial was performed. The results are shown in **Figures 5B,C**. We observed that the time spent in the target quadrant [$F(2, 26) = 6.443$, $p < 0.01$] and the number of crossings over the previous location of the escape platform [$F(2, 26) = 4.21$, $p < 0.05$] were both significantly lower in the MS + CRS group than in the control group, but no difference was observed between the CRS and control groups.

Effects on Hippocampal PSD95 and Synaptophysin mRNA and Protein Levels

The PSD95 and synaptophysin mRNA levels in the hippocampus were measured by quantitative RT-PCR. The relative target

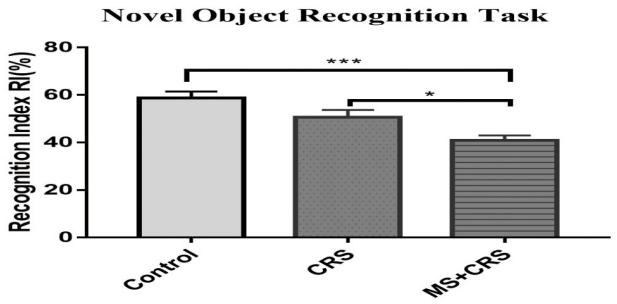

FIGURE 4 | Behavioral changes in the novel object recognition task (NORT). Performance in the NORT shown by the recognition index. Error bars, SEM; n = 9–10 mice in each group. $^{*}P < 0.05$, $^{***}P < 0.001$.

gene mRNA levels in the groups are shown in **Figure 6**. As shown in **Figures 6A,B**, the expression levels of PSD95 mRNA in the hippocampus were lower in the mice in the CRS and MS + CRS groups than in the mice in the control group [PSD95: $F(2, 9) = 10.856$, $P < 0.05$, $P < 0.01$; synaptophysin: $F(2, 9) = 17.920$, $P < 0.05$, $P < 0.001$].Tukey's *post-hoc* tests revealed that the levels of synaptophysin mRNA in the MS + CRS group were significantly lower than those in the CRS group ($p < 0.05$). However, the levels of PSD95 mRNA between the CRS and MS + CRS groups showed no significant difference; although the results from the MS + CRS group showed a trend toward a reduction compared with the CRS group, this difference did not reach statistical significance ($P > 0.05$).

Changes in PSD95 and synaptophysin protein expression are shown in **Figures 6C,D**. One-way ANOVA revealed that the CRS and MS + CRS groups had significantly lower PSD95 and synaptophysin protein levels than the control group [PSD95: $F(2, 9) = 18.441$, $P < 0.05$, $P < 0.001$; synaptophysin: $F(2, 9) = 26.074$, $P < 0.05$, $P < 0.001$]. Moreover, Tukey's *post-hoc* test demonstrated that the MS + CRS group had significantly lower PSD95 and synaptophysin protein levels than the CRS group

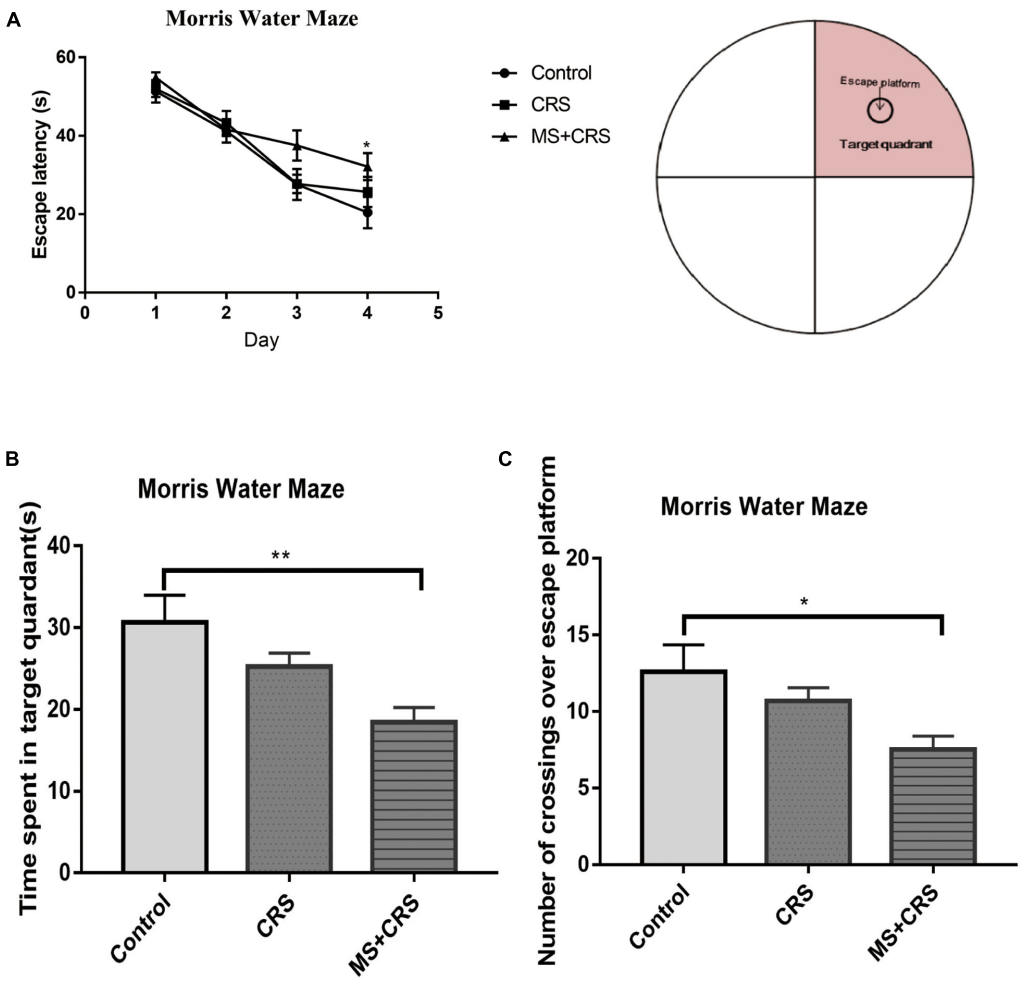

FIGURE 5 | Behavioral changes in the Morris water maze (MWM). **(A)** Escape latency time during the acquisition trial. **(B)** Time spent in the target quadrant during the probe trial. **(C)** Number of crossings over the previous location of the escape platform during the probe trial. Error bars, SEM; n = 9 mice in each group. $^{*}P < 0.05$, $^{**}P < 0.01$.

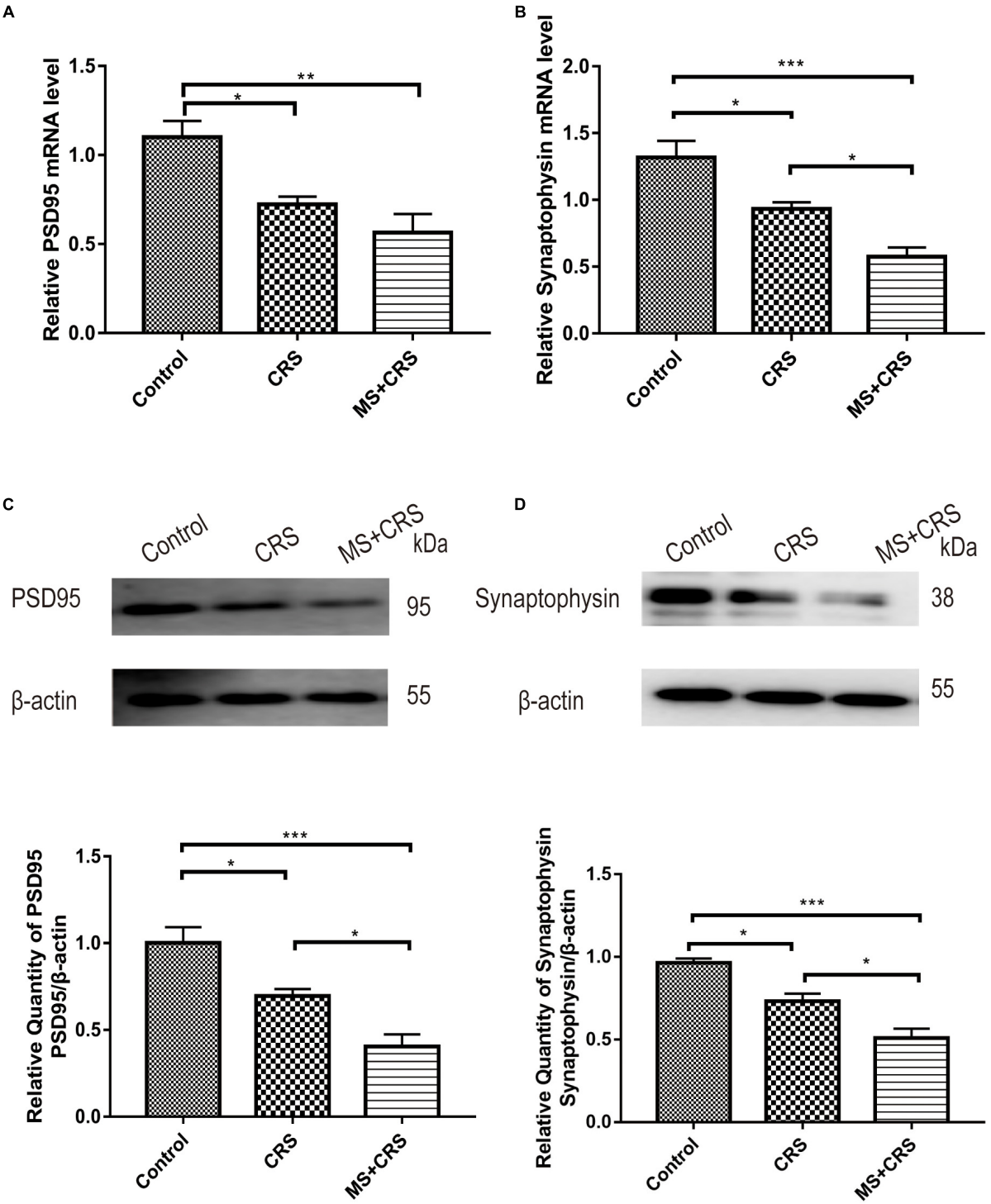

FIGURE 6 | PSD95 and synaptophysin mRNA and protein expression levels in the hippocampus. mRNA expression levels were measured by RT-qPCR, and the experiment was repeated twice. Quantitative analysis was normalized to GAPDH. Protein expression levels were determined by Western blotting analysis. **(A)** PSD95 mRNA expression ($n = 4$). **(B)** Synaptophysin mRNA expression ($n = 4$). **(C)** PSD95 protein expression ($n = 4$). **(D)** Synaptophysin protein expression ($n = 4$). Gel images from Western blotting. Data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

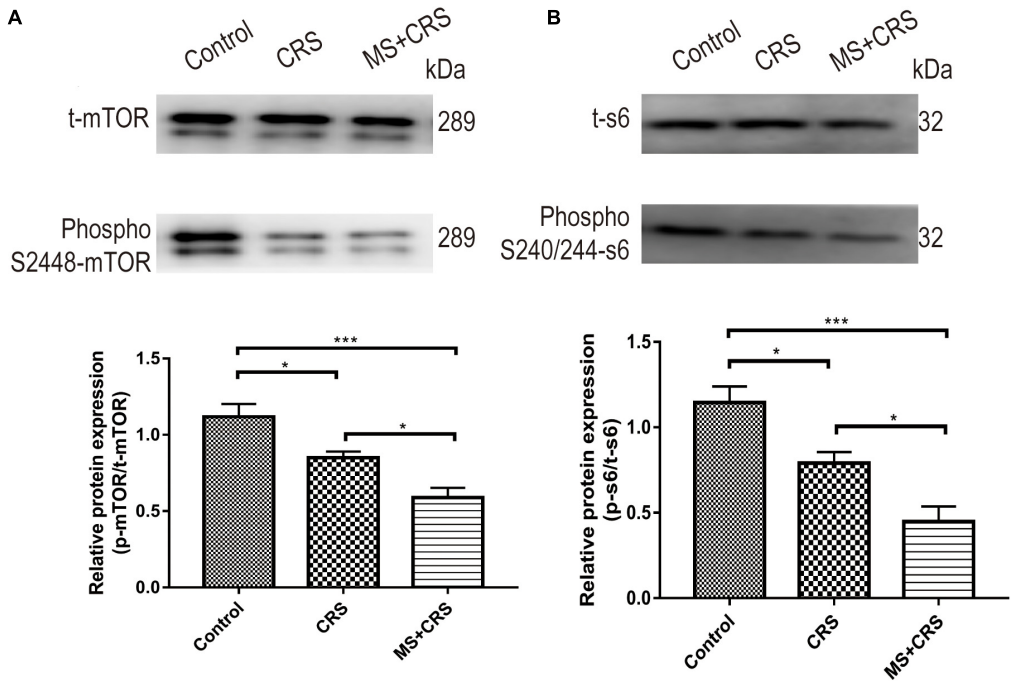

FIGURE 7 | p-mTOR/t-mTOR and p-s6/t-s6 protein expression levels in the hippocampus. **(A)** p-mTOR/t-mTOR protein expression (n = 4). **(B)** p-s6/t-s6 protein expression (n = 4). Gel images from Western blotting. Data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$.

($P < 0.05$, both). Whole-gel images from the Western blotting are provided in **Figures 6C,D**.

Effects on the Hippocampal mTOR-s6 Pathway

We observed an appreciable reduction in phospho-mTOR (S2448) and phospho-s6 (S240/244) in the CRS and MS + CRS groups compared with the control group [phospho-mTOR/t-mTOR: $F(2, 9) = 16.528$, $P < 0.05$, $P < 0.001$; phospho-s6/t-s6: $F(2, 9) = 17.193$, $P < 0.05$, $P < 0.001$], but the total mTOR and s6 levels remained unaltered. Reduced phosphorylated levels of mTOR and s6 protein in the CRS and MS + CRS mice indicated that the mTOR-S6 pathway was inhibited. As further evidence, the results showed that the levels of phospho-mTOR and phospho-s6 were significantly lower in the MS + CRS group than in the CRS group ($P < 0.05$) (**Figure 7**).

DISCUSSION

It is well-known that early adverse stress can exert a harmful influence on brain development, behavior and neuroplasticity and lead to psychopathology and cognitive impairments that can persist until adulthood. The occurrence of mental illnesses is closely associated with adverse experiences in childhood (Saleh et al., 2017). In addition, some research has suggested that adults with major depression and ELS are more difficult to treat than those who had not been exposed to ELS (Targum and Nemeroff, 2019). The aim of the present study was to explore whether MS during early life can aggravate the negative impact of stress later in life. This study showed that ELS increased susceptibility to stress later in life and induced more serious anxiety-like symptoms and cognitive impairments. Moreover, our results suggested that MS decreased the expression of PSD95,synaptophysin, phospho-mTOR, and phospho-s6 in the mouse hippocampus.

Maternal Separation Increased Anxiety-Like Behavior and Impaired Recognition Function

It has been reported that maternally separated rats showed anxiety- and depressive-like behaviors in adulthood (Fodor et al., 2012; Vetulani, 2013). Many previous studies have reported that CRS induces anxiety and depression in rodents (Campos et al., 2013; Jangra et al., 2020). Therefore, the CRS model has been widely applied in the study of mental illness. In this study, increasing anxiety-like behaviors in the CRS group were also observed. However, it has rarely been reported whether MS exaggerates the anxiety-like behavior of animals following CRS. In present study, we used the OFT and EPM test to assess the anxiety-like behaviors of mice and showed that mice in the MS + CRS group displayed more anxiety-like behavior than those in the CRS group, as evidenced by decreased time spent in the open arms of the EPM and central area during the OFT. This study not only found that CRS induced anxiety-like behavior but also suggested that MS exacerbated the negative effect of CRS. These changes suggested that MS exposure increased sensitivity to stress and led to more serious anxiety-like behaviors

than non-exposure to MS in mice. These results concur with clinical research data showing that exposure to early adverse experiences increases the probability of developing anxiety and depression later in life (Heim and Nemeroff, 2001). Thus, ELS aggravates anxiety-like behavior and increases vulnerability to stress later in life.

On the other hand, previous studies have reported that after a protocol of ELS, recognition in animals is impaired (Aisa et al., 2007; Ivy et al., 2010). We found that mice subjected to MS and restraint stress displayed cognitive impairments in the NORT and MWM tests, but not in the CRS group. More specifically, the MS + CRS group was significantly different compared to the control group, but no significant difference in spatial acquisition learning and memory in the MWM was observed between the CRS and control mice. The NORT data also showed that the RI value in the MS + CRS group was lower, indicating that non-spatial learning and memory were also disrupted, although this was not the case in the CRS group. Those results suggested that MS can impaired recognition function. But some studies have claimed that MS does not affect spatial memory in the MWM or that spatial memory was enhanced (Enthoven et al., 2008; Banqueri et al., 2017). These differences may be caused by different MS protocols and animal strains. In addition, no significant disruption in learning and memory was observed in the mice of CRS group in our study, which is in disagreement with previous studies using CRS model (Abidin et al., 2004; Perez et al., 2018). In our present study, we applied chronic restraint stress procedure at adolescence rather than at adulthood. Some reports revealed that effects of adolescent-stress may be delayed (Isgor et al., 2004), which is different from adult-stress. Therefore, we speculated that the delayed effect of adolescent-stress may be the reason for no significant deficits in cognitive functions in CRS group mice compared to control, but it does not mean that CRS has no effect on recognition function.

Maternal Separation Downregulates the Level of Synaptic Proteins

It has been reported that PND2–PND21 is a key period for hippocampal development. Exposure to ELS during this period may lead to long-term changes in synaptic plasticity (Derks et al., 2016). Synaptic plasticity in the hippocampus is closely related to learning and memory and the onset of multiple mental illnesses, including depression and anxiety (Duman et al., 2016). Reduced synaptic plasticity is not conducive to appropriate adaptive responses to subsequent stress. Promoting synaptic plasticity in the hippocampus can improve spatial learning and memory (Li et al., 2019). It is well-known that impairments in synaptic plasticity are associated with the downregulation of synaptic proteins. Therefore, we detected the levels of synaptic proteins in presynaptic and postsynaptic membranes, including PSD95 and synaptophysin, which are closely related to synaptic plasticity. The experimental results showed that the mRNA and protein expression levels of PSD95 and synaptophysin in the hippocampus were lower in the MS + CRS mice than in the CRS mice. These results revealed that MS reduced synaptic proteins and had a negative effect on synaptic function and plasticity. This finding is in line with previous studies showing a downregulation in PSD95 protein expression in maternally separated rats (Ganguly et al., 2015). Thus, this finding suggests that anxiety-like behavior and recognition deficits in maternally separated mice are related to down regulation of the expression of hippocampal synaptic proteins.

Maternal Separation Inhibits the mTOR-S6 Pathway

In the present study, MS significantly reduced synaptic protein levels of PSD95 and synaptophysin. It has been confirmed that MS leads to a deficiency in synaptic protein translation and has detrimental effects on localized *de novo* activity-induced synaptic protein translation (Ahmad et al., 2018). Alterations in synaptic protein translation are regarded as important facets of neuronal pathologies and neuropsychiatric disorders (Li et al., 2010; Wang et al., 2010). The activity of mTOR regulates translation initiation. Definitive evidence has suggested that mTOR is closely related to the process of synaptogenesis. Activation of mTOR leads to phosphorylation of s6, thereby upregulating the expression of PSD95 and synaptophysin and promoting the production of new synapses (Duman et al., 2012). The mTOR-s6 pathway can potentiate synaptic transmission (Luft et al., 2004) by facilitating the synthesis of synaptic proteins (Bhattacharya et al., 2012). Activation of the mTOR-s6 pathway contributes to synthesis of postsynaptic protein proteins (Tavares et al., 2018). Antidepressants activate the mTOR/s6 kinase signaling pathway and increase the expression of synaptic proteins (Tavares et al., 2018). In this study, the activation of mTOR-s6 was investigated to determine the underlying molecular mechanisms. Our results showed that the levels of phosphorylated mTOR and s6 were significantly decreased in the hippocampus of MS + CRS mice compared to CRS and control mice. This shows that ELS reduced mTOR-s6 signaling activity in the hippocampus.

In summary, we speculate that the mechanisms underlying synaptic plasticity following MS are possibly associated with mTOR-s6 signaling. Notwithstanding, this study has certain limitations. The effects of mTOR inhibitors, such as rapamycin, were not evaluated in the present study. Thus, additional work needs to be done to address these limitations in the future.

CONCLUSION

In conclusion, we found that maternal separation aggravated anxiety-like behavior and cognitive deficits and disrupted synaptic plasticity. The mechanisms of these effects may be related to mTOR-s6 pathway inhibition. This implies that clinical treatments of individuals exposed to early-life stress should be different from those without early adverse experiences.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at the Jinan University (Approval No. IACUC-20190702-03).

AUTHOR CONTRIBUTIONS

AW, JC, and XZ conceived and designed the experiments. AW and XL performed the research and wrote the manuscript. JW and NY contributed to the animal experiments. AW, QM, and XL contributed to the conducted molecular experiments. NY, JW, and XZ analyzed the data. JC funded the research and revised the manuscript. All authors read and approved the final manuscript.

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frontiers in Genetics

REVIEW
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Genetic and Epigenetic Consequence of Early-Life Social Stress on Depression: Role of Serotonin-Associated Genes

Tomoko Soga, Chuin Hau Teo and Ishwar Parhar*

Brain Research Institute, Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway, Malaysia

Early-life adversity caused by poor social bonding and deprived maternal care is known to affect mental wellbeing and physical health. It is a form of chronic social stress that persists because of a negative environment, and the consequences are long-lasting on mental health. The presence of social stress during early life can have an epigenetic effect on the body, possibly resulting in many complex mental disorders, including depression in later life. Here, we review the evidence for early-life social stress-induced epigenetic changes that modulate juvenile and adult social behavior (depression and anxiety). This review has a particular emphasis on the interaction between early-life social stress and genetic variation of serotonin associate genes including the serotonin transporter gene (5-HTT; also known as SLC6A4), which are key molecules involved in depression.

Keywords: serotonin, depression, social stress, epigenetic modification, 5-HT receptor

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****Correspondence:***
Tomoko Soga
tomoko.soga@monash.edu

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INTRODUCTION

History of early-life social stress indicates adverse effects on functions of the hypothalamic-pituitary-adrenal axis and stress response in later life (Denhardt, 2018; Lapp et al., 2019) linked to the development of the major depressive disorder in adolescents and adults (Hettema et al., 2006; Pace et al., 2006; Rao et al., 2008; Heim and Binder, 2012; Bunea et al., 2017). These clinical findings are backed by animal studies demonstrating that poor social bonding and reduced maternal care can subsequently cause altered behavior and heightened anxiety, as well as negative consequences on the brain development of offsprings (Eiland and McEwen, 2012; Carini and Nephew, 2013; Murgatroyd et al., 2015).

The effect of early-life social stress on the genetics of depression can be described as the influence of the environment on the genes of the brain – in other words, epigenetics. Epigenetics involves modifications to gene expression that may be inherited by the offspring, without any changes in the DNA sequences that encodes for those genes (Hochberg et al., 2011). Epigenetic modifications involve three processes, DNA methylation, histone modification, and various RNA-mediated processes. In DNA methylation, a methyl group is transferred to C-5 of a cytosine residue in DNA – this interferes with the ability of transcription factors to bind to DNA, and as such, high methylation levels are associated with repression of gene expression (Crabtree, 2020). Histone modification, on the other hand, involves either methylation, acetylation, or phosphorylation of amino acids in the

histone protein tails; as the histones control how tightly chromatin is coiled and a tightly packed chromatin restricts access of regulatory factors to DNA, modification of those histones can control how much genes are expressed (Crabtree, 2020). Finally, non-coding RNAs can facilitate chromatin modifications, while microRNAs can pair to complementary target mRNAs, directly suppressing translation from mRNA to protein (Crabtree, 2020).

It is well known that early-life social stress leads to persistent epigenetic modifications of target genes associated with changes in emotional behavior (Nugent et al., 2011; McCann et al., 2017; Fogelman and Canli, 2019). There has been a growing body of work in the past decade, documenting epigenetic action in the brain, stemming from exposure to early-life social stress in animal models and human studies (Murgatroyd et al., 2009; McClelland et al., 2011; Heim and Binder, 2012; Huang, 2014; Provençal and Binder, 2015; Vaiserman, 2015). These studies have indicated various lasting changes in gene expression due to early-life stress, such as altered arginine vasopressin expression (Murgatroyd et al., 2009) and increases in seizure and epilepsy incidences (Huang, 2014). Prenatal stress caused elevated methylation of a glucocorticoid receptor gene in infants, altering their reactivity to stress (Oberlander et al., 2008). A genome-wide study discovered significant methylation differences within promoters of subjects exposed to early-life stress; 248 promoters showed hypermethylation, while 114 showed hypomethylation. The expressions of genes involved in neuronal plasticity, in particular, were significantly different (Labonté et al., 2012). Genes undergoing methylation in association with early-life social stress-induced depression are also well studied (Schoenherr and Anderson, 1995; Jiang et al., 2019). The monoaminergic theory is supported as the main neuropathogenesis of depression. Based on this, epigenetic modifications of monoamine-related genes such as transporters, metabolic enzymes, synthesis enzymes, and receptors have been well investigated to understand the neuropathogenesis of depression.

Serotonin (5-hydroxytryptamine, 5-HT) is the main monoamine system involved in the neuropathogenesis of depression. The key 5-HT-related genes are serotonin transporter (5-HTT; also known as SLC6A4), monoamine oxidase A (MAO-A), tryptophan hydroxylase 2 (TPH2), and 5-HT receptors. These 5-HT-related genes and their signaling pathways are involved in brain development, stress response, and emotional control. Epigenetic alterations of 5-HT-related genes may be the underlying effect of early life stress on depression (Parade et al., 2017). Since depression is closely tied to the 5-HT system, therefore, the examination of epigenetic influence on 5-HT-associated genes could generate an interesting body of work that would serve better to explain the interplay between nature and nurture in depression. In this review, we looked at the mounting evidence for early life social stress-induced epigenetic changes in several 5-HT-associated genes and how these gene modifications influence behavior in later life. Besides, treatments that reduce early-life social stress are also reviewed to understand their impact on the attenuation of genetic and behavioral changes.

EARLY LIFE SOCIAL STRESS-INDUCED EPIGENETIC CHANGES IN SEROTONIN-RELATED GENES

Serotonin Transporter

The 5-HTT gene was first sequenced and characterized by Lesch et al. (1994), but its function in terminating serotonergic neurotransmission was already documented earlier (Kanner and Schuldiner, 1987). Variants of 5-HTT gene potentially increase susceptibility to a stressful environment, increasing the risk for mental disorders. There have been numerous studies and reviews covering the role of 5-HTT in psychiatric disorders, indicating that polymorphisms in the 5-HTT lead to serotonergic dysfunction that can develop into various diseases such as major depressive disorder and bipolar disorder (Mann et al., 2000; Zanardi et al., 2000; Hahn and Blakely, 2002; Anguelova et al., 2003; Murphy et al., 2004; Abdolmaleky et al., 2014). An epidemiology study performed on a cohort of 1,037 children identified a correlation between the presence of a functional polymorphism in the 5-HTT gene in individuals having a heterozygous or homozygous variant of the short allele, who exhibited greater susceptibility to life stress history for predicting depression, compared to individuals with a homozygous long allele variant (Caspi et al., 2003).

The influence of epigenetics on 5-HTT is also well documented. For example, DNA hypermethylation, an indicator of epigenetic influence, has been observed in the 5-HTT gene of schizophrenic patients alongside reduced 5-HTT expression (Abdolmaleky et al., 2014). Increased methylation in the proximal promoter region of 5-HTT is an epigenetic change that has a positive correlation with increased responsiveness to threat in the amygdala (Nikolova et al., 2014).

As stress is one of the major drivers for epigenetic changes in the brain (Gudsnuk and Champagne, 2012), it stands to reason that early life social stress would also have a major effect on epigenetic modifications to the 5-HTT gene. Methylation of retro-transposonal AluJb element is associated with stress response under major depressive disorder, where lower methylation has a better stress-adaptive reaction (Schneider et al., 2018). The link between methylation and depression is influenced by genetic variation – specific genotypes have higher methylation associated with depression, while those homozygous for short 5-HTT alleles exhibit lower methylation in association with depression (Lam et al., 2018). DNA methylation of 5-HTT in the 10-year-old twins experiencing discordant stress through bullying has been demonstrated to be significantly higher compared to their co-twin that did not undergo bullying in the same period (Ouellet-Morin et al., 2013). The focus on twins indicates that childhood bullying – early life social stress – is the influencer for increased 5-HTT methylation rather than predetermined genetic factors. The bullied twin also exhibited blunted cortisol responses in comparison to the non-bullied twin (Ouellet-Morin et al., 2013).

Research using animal models supports the role of stress during early life – it causes epigenetic changes that may be associated with risk of depression. Peer rearing in rhesus

macaques, which is a form of early life stress in comparison to maternal rearing (Harlow and Suomi, 1974; Suomi et al., 1976), causes reduced H3K4me3 (Histone 3 protein with trimethylation at lysine 4) binding at the promoter of the 5-HTT gene (Lindell et al., 2012). As H3K4me3 is an epigenetic modification that promotes gene expression, lower H3K4me3 indicates lower 5-HTT expression. This is further supported by the finding of serotonin metabolite 5-HIAA in the cerebrospinal fluid of peer-reared macaques, which suggests a decreased serotonergic function in the central nervous system (Lindell et al., 2012).

Homozygous and heterozygous 5-HTT knockout rats when exposed to early-life stress show decreased serotonergic innervation to Edinger-Westphal urocortin 1 neurons (van der Doelen et al., 2017). Abnormal levels of urocortin 1 have been associated with major depressive disorder (Ryabinin et al., 2012; Waters et al., 2015), suggesting that early-life stress can interact with 5-HTT to cause depressive-like neurophysiology. Furthermore, heterozygous 5-HTT knockout rats exposed to early life social stress triggered by maternal separation, exhibit anhedonic behavior in the form of lower sucrose preference (Houwing et al., 2019). The same study also found low gene expression of nerve growth factor. Examination of clinical studies has reported a significant correlation between reduced nerve growth factor expression and the diagnosis of major depressive disorder (Chen et al., 2015). As a whole, these studies provide strong support for the role of epigenetic action on 5-HTT in early life social stress.

Monoamine Oxidase A

MAOA is involved in breaking down serotonin. An increase in MAOA expression results in a decrease in serotonin levels in the brain, which has been suggested as the main factor in major depressive disorder (Naoi et al., 2018). Epigenetic regulation of MAOA has been documented in humans (Shumay et al., 2012). In particular, methylation of MAOA in the promoter region of CpG5 and CpG11 increases MAOA expression, which in turn decreases serotonin levels; this has been observed in female patients with depression (Domschke et al., 2015). Behavioral disinhibition in children has been associated with a functional promoter polymorphism on *MAOA* (*MAOA-LPR*) that interacts with early life social stress (Enoch et al., 2010). Furthermore, while early life social stress has been associated with increased aggressive disorders in males through the *MAOA-L* allele, such stress exposes *MAOA-L* females to a higher risk of developing depression (Melas et al., 2013). It has been suggested that the susceptibility of *MAOA-L* females to depression may be a result of epigenetic dysregulation of *MAOA* by early life stressors, which affects DNA methylation of the glucocorticoid receptor gene *NR3C1* (Melas et al., 2013).

Studies in rodents have also drawn similar connections between early life stress and epigenetic control of MAOA. Early life social stress induces CpG-specific methylation in the *MAOA* promoter, which elevates MAOA expression in the dorsal striatum – this is associated with voluntary alcohol consumption (Bendre et al., 2019). The effect of peripubertal stress on the epigenetic state of *MAOA* is associated with the development of antisocial behavior (Márquez et al., 2013). The development of aggressive behavior is sexually dimorphic, with MAOA hypermethylation in the hypothalamus and in the prefrontal cortex of male rats. In contrast, female rats do not exhibit any changes in epigenetic control of *MAOA* (Konar et al., 2019).

Tryptophan Hydroxylase 2

The gene for tryptophan hydroxylase 2 (TPH2) is a neuron-specific rate-limiting 5-HT biosynthetic enzyme in the brain. Alterations in *TPH2* gene expression is involved in the pathogenesis and treatment of MDD (Tsai et al., 2009; Xu et al., 2012). Single-nucleotide polymorphisms (SNPs) in *TPH2* gene are linked to 5-HT dysfunction (Gao et al., 2012), which have been associated with MDD (Zill et al., 2004; Zhang et al., 2005), and one of the SNPs in the TPH2 gene is associated with amygdala and hippocampal volume (Inoue et al., 2010). The promoter region of the *TPH2* gene lacks a CpG island; however, there are numerous scattered CpG sites and an enriched signal of DNA hypomethylation at the 5′-UTR locus (Chen and Miller, 2012). A recent study has shown that hypermethylation of the CpG-site in the *TPH2* gene during early-life stress could reduce antidepressant response within the first 2 weeks of treatment in patients with MDD (Xu et al., 2016; Shen et al., 2020). Furthermore, methylation of a single CpG site in the promoter region of TPH2 significantly decreases *TPH2* gene expression levels. This methylation is also partially linked with suicide in MDD patients (Zhang et al., 2015). These studies suggest the impact of early life social stress-associated epigenetic action on *TPH2* gene and depression.

5-HT Receptors

Seven families of 5-HT receptors and their subtypes have been identified, namely, 5-HTI (5-HT1A, 5-HT1B, 5-HTID, 5-HTIE, and 5-HT1F), 5-HT2 (5-HT2A, 5-HT2B, and 5-HT2C), 5-HT3, 5-HT4, 5-HT5 (5-HT5A and 5-HT5B), 5-HT6, and 5-HT7. 5-HT receptor-specific agonists and antagonists have been designed and developed as therapeutics against mental disorders. Among the 5-HT receptors, the most well-studied receptor is 5-HT1A, known as an autoreceptor, which has inhibitory control over the 5-HT neuronal activity. Increased levels of 5-HT1A in 5-HT neurons of the dorsal raphe have been reported in MDD patients and suicide victims with MDD (Stockmeier et al., 1998; Hesselgrave and Parsey, 2013). 5HT1A is also a postsynaptic receptor, expressed in main target brain areas, the hippocampal, cortical, and hypothalamic regions, that are associated with depression, stress, and anxiety (Albert et al., 2019). Several SNPs and stress-induced DNA methylation of the 5-HT1A promoter have been associated with MDD and alteration in their response to antidepressants. C(−1,019)G (rs6295) is a functional 5-HT1A promoter polymorphism that modifies *5-HT1A* gene expression in a brain region-specific manner (Le Francois et al., 2008) and modify connectivity such as amygdala-ventrolateral prefrontal cortex, and corticolimbic connectivity related to MDD (Vai et al., 2017). In fact, C(−1,019)G (rs6295) promoter

polymorphism in 5-HT1A elevated risk of depression (Benedetti et al., 2011; Kim et al., 2011; Vai et al., 2017), resistance to an antidepressant (Wang et al., 2018b), panic disorder (Choe et al., 2013), fear (Straube et al., 2014), gender-dependent modulatory effects on depression, physical function in patients with pain (Lebe et al., 2013), and suicidal attempt in MDD (Sawiniec et al., 2007). Kim and co-workers have reported interactions between C-1019G polymorphism in 5HT1A and negative life stressors that account for MDD symptoms (Kim et al., 2011). These findings support those genetic alterations of the 5-HT1A promoter that make it sensitive to stress and increase the risk of MDD.

Some studies have suggested that human *5HT1A* gene methylation is associated with MDD. Increased DNA methylation of 5HT1A promoter in leukocytes has been reported in bipolar depression (Carrard et al., 2011). Stress-linked hypomethylation of CpG668 site in the *5HT1A* gene from blood samples is associated with resistance to antidepressants in treatment-naive MDD patients (Wang et al., 2018a).

Studies in animal models suggest that early-life social stress induces persistent changes in 5-HT1A expression levels in the amygdala, hippocampus, and dorsal raphe nucleus (Bravo et al., 2014). Furthermore, early-life stress, in combination with adult social isolation, dramatically decreases the 5-HT1A-mediated inhibition of layer II/III pyramidal neuronal activities (Goodfellow et al., 2009). Le Francois and co-workers have reported methylation of 24 CpG sites on the mouse 5-HT1A promoter, and chronic mild stress increased DNA methylation of a single site located within the Sp4 element of the *5HT1A* gene that correlates with increased mRNA expression levels in the raphe and prefrontal cortex in male mice (Le Francois et al., 2015). In brief, subjects with methylation of the *5-HT1A* gene variant may be more susceptible to developing MDD.

Other variants of 5-HT receptors have also been reported to have a risk of MDD. Methylation of 5-HTR2A genotype at two CpG sites (−1,420 and −1,224) has been associated with PTSD and MDD under contextual stress (Parade et al., 2017). An SNP in the allele of −1438A/G (rs6311) in the 5HTR2A promoter is highly influenced by genetic factors and the environment in female MDD patients (Lebe et al., 2013). These studies support that 5HT2A methylation is a mechanism by which early adversity is biologically encoded. In another case, epigenetic modification of the 5-HT3A is involved in the molecular mechanism underlying the relationship between childhood maltreatment and the severity of neuropsychiatric diseases in adulthood (Perroud et al., 2016). These studies of epigenetic regulation of 5-HT and 5-HT receptors could be applied for more effective personalized treatments for MDD.

Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) is a neurotrophin involved in many of the brain's activities, including, but not limited to, neuronal development, synaptic modulation, and plasticity, as well as hippocampal function (McAllister et al., 1999; Huang and Reichardt, 2001; Lu, 2003; Monteggia et al., 2004). While BDNF plays a role in serotonergic expression, it may also itself be regulated by 5-HT, particularly in depression and stress (Martinowich and Lu, 2008). During the depression, the role of BDNF can vary; in the hippocampus and the prefrontal cortex, BDNF expression is associated with inhibition of depressive symptoms, whereas it promotes anxiety-like symptoms in the nucleus accumbens and the amygdala (Yu and Chen, 2011). Higher DNA methylation of the *Bdnf* gene has been associated with the improved antidepressant response, with escitalopram treatment increasing methylation after 8 weeks (Wang et al., 2018b).

The effect of early life stress on BDNF has been well studied in the past decade. Maltreatment of rat pups by stressed caretakers during infancy elicited significant methylation of *Bdnf* exons in the prefrontal cortex – the presence of methylation persists even into adulthood, demonstrating a long-term effect (Roth et al., 2009). The postnatal maternal separation was found to induce a decrease in exon IV *Bdnf* mRNA, with adult restraint stress further exacerbating the maternal separation-induced drop in BDNF expression (Seo et al., 2016). Furthermore, *Bdnf* promoter IV displays a decrease in acetylation of histone 3 (H3) and histone 4 (H4) in adult restraint stress, and further reduction in acetylation of H3 and H4 is observed from maternal separation. However, these epigenetic changes can be recovered by escitalopram treatment (Seo et al., 2016).

In addition to postnatal maternal separation, which occurs at a very young age, adolescent social stress in mice also causes epigenetic changes to BDNF in adulthood (Xu et al., 2018). Here, *Bdnf* gene expression is downregulated in the medial prefrontal cortex as a result of adolescent social stress, and increased dimethylation of H3 at lysine 9 (*H3K9me2*) downstream of the *Bdnf* IV promoter – his occurs in conjunction with cognitive flexibility in the mice after reaching adulthood. Both epigenetic changes and behavioral changes can be reverted by antidepressant treatment (Xu et al., 2018). The effect of maternal separation on behavior has been examined in a similar study; early-life interaction with a stranger can induce a stressful social experience, and as a result, less social interaction with strangers is observed from pups who have been separated from their mothers (Karen and Rajan, 2019). Furthermore, the stressful social experience subsequently elevated DNA methyltransferase (*Dnmt3a*) as well as other epigenetic elements such as decreased acetylation and increased methylation of histones in the amygdala of rats, which had been raised under maternal separation (Karen and Rajan, 2019).

In addition to stress-induced epigenetic changes, a recent study also investigates the effect of early life stress across generations and gender. By subjecting the first generation of rats to maternal separation and the subsequent generation raised in a balanced cross-fostering manner, it was found that early-life stress through maternal separation resulted in increased *Bdnf* methylation in both male and female rats, but *Bdnf* expression was reduced only in females (Coley et al., 2019). Subsequently, the second-generation rats from an early life social stress lineage exhibited increased *Bdnf* methylation, while fostered female rats raised by a first-generation mother, which had previously undergone early life stress exhibited *Bdnf* methylation (Coley et al., 2019).

These studies suggest that stress-induced epigenetic changes are carried across generations.

TREATMENTS ATTENUATING EARLY-LIFE SOCIAL STRESS CHANGES

While early-life social stress may induce adverse epigenetic and behavioral changes, these changes might not be entirely irreversible. As noted above, the use of antidepressants such as escitalopram has proven effective in recovering epigenetic changes in the *Bdnf* gene in both humans and rats (Seo et al., 2016; Wang et al., 2018b). Lithium treatment has been noted to reverse the effects of early-life social stress by increasing neuropeptide Y and corticotropin-releasing hormone – both associated with depression and stress vulnerability – in the adult rat hypothalamus (Husum and Mathé, 2002). Valproic acid treatment helps treat cognitive dysfunction induced by amphetamine to mimic a later-life social stress event. Still, a combination of both early life stress and later life stress renders the treatment ineffective (Pinheiro et al., 2012). Other treatments attenuating early-life social stress changes can be found in corticotropin-releasing hormone blockers, which recover early-life social stress-induced hippocampal dysfunction (Ivy et al., 2010), or the use of dopamine receptor 3 (Drd3) agonists to increase dopaminergic neuronal activity, which has been shown to restore normal social behavior in mice that have undergone early-life social stress (Shin et al., 2018).

LIMITATIONS AND FUTURE PERSPECTIVES

While the role of epigenetics in regulating 5-HT-associated genes under early-life social stress appears to be backed by substantial evidence, limitations remain when it comes to translating those results to clinical research; after all, the same methods used to directly study the expressions of genes in the rodent brain cannot be effectively implemented with humans. The relative scarcity of neurological data available means that investigation of peripheral expression levels for genes such as 5-HTT (Olsson et al., 2010) and BDNF (Lopez et al., 2013) in association with epigenetic regulation under exposure to early life stress is necessary for future research. Furthermore, future studies may also want to consider and compare the effect of the social and natural environment on epigenetic regulation; stress in early life can come through various means, and as such, natural obstacles such as food deprivation may yet generate different responses compared to social stress.

Even so, epigenetic studies have proven to be highly useful in improving our understanding of the biological processes that serve as the fundament for social influences on health. By combining human epidemiological studies and animal model experimental studies, the role of epigenetic mechanisms in social stress-related health risks should become clearer. This would help advise public health and social interventions, which serve to reduce epigenetic aging and improve long-term health.

SUMMARY

Early life social stress may be a driving force for susceptibility to depression in later years, and epigenetic regulation of serotonin-associated genes is another means by which early-life social stress exerts its influence (**Figure 1**). Genetic changes to the associated genes in later life have proven to be a strong indicator for depressive disorders in both animal models and clinical studies – this suggests that targeted recovery of these

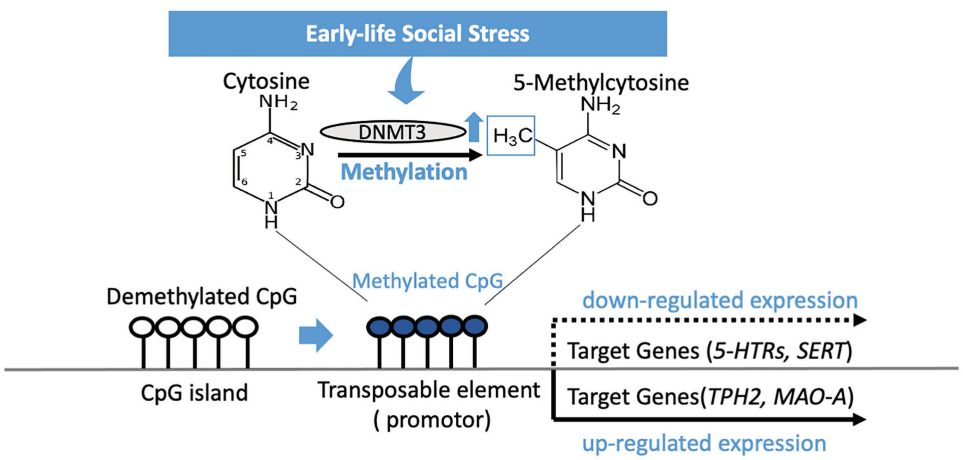

FIGURE 1 | DNA methylation of CpG islands in the genetic code silences expression of a gene after early-life social stress exposure. Methylation occurs by a methyltransferase (DNMT3) transferring a methyl group to cytosine, and when occurring on a transposable element has the effect of repressing gene transcription related to that element. *5-HTRs*, 5-HT receptors; *SERT*, serotonin transporter; *TPH2*, tryptophan hydroxylase 2; *MAO-A*, *monoamine oxidase* A.

epigenetic changes is a potential path to take when considering treatment of the major depressive disorder. Epigenetic changes to serotonin-associated genes are tied directly to increased or decreased genetic expression, which in turn is correlated to behaviors distinctive of depressive disorders. However, one thing of note when it comes to epigenetic changes in the sexual dimorphism present in their effects is an observation that is recurring in both BDNF and MAOA, where the presence of the epigenetic changes might be opposed or non-existent depending on the sex. Regardless, as progress in epigenetics advances, greater understanding and better treatment philosophies for depression may arise in the future.

AUTHOR CONTRIBUTIONS

TS and IP designed the flow of this review paper and edited the manuscript. TS and CT wrote the main manuscript and prepared a figure. All authors contributed to the article and approved the submitted version.

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Gene Transcript Alterations in the Spinal Cord, Anterior Cingulate Cortex, and Amygdala in Mice Following Peripheral Nerve Injury

Songxue Su[1,2†], Mengqi Li[2,3†], Di Wu[4], Jing Cao[1,2], Xiuhua Ren[1,2], Yuan-Xiang Tao[5] and Weidong Zang[1,2*]*

[1] Department of Anatomy, College of Basic Medicine, Zhengzhou University, Zhengzhou, China, [2] Neuroscience Research Institute, Zhengzhou University Academy of Medical Sciences, Zhengzhou, China, [3] Department of Anesthesiology, Pain and Perioperative Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [4] Department of Bioinformatics, College of Life Sciences, Zhengzhou University, Zhengzhou, China, [5] Department of Anesthesiology, Rutgers New Jersey Medical School, The State University of New Jersey, Newark, NJ, United States

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Edited by:
Fang Pan,
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Reviewed by:
Tao Chen,
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Ying Xu,
University at Buffalo, United States

****Correspondence:***
Yuan-Xiang Tao
yuanxiang.tao@njms.rutgers.edu;
yt211@njms.rutgers.edu
Weidong Zang
zwd@zzu.edu.cn

[†] These authors have contributed equally to this work

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Chronic neuropathic pain caused by nerve damage is a most common clinical symptom, often accompanied by anxiety- and depression-like symptoms. Current treatments are very limited at least in part due to incompletely understanding mechanisms underlying this disorder. Changes in gene expression in the dorsal root ganglion (DRG) have been acknowledged to implicate in neuropathic pain genesis, but how peripheral nerve injury alters the gene expression in other pain-associated regions remains elusive. The present study carried out strand-specific next-generation RNA sequencing with a higher sequencing depth and observed the changes in whole transcriptomes in the spinal cord (SC), anterior cingulate cortex (ACC), and amygdala (AMY) following unilateral fourth lumbar spinal nerve ligation (SNL). In addition to providing novel transcriptome profiles of long non-coding RNAs (lncRNAs) and mRNAs, we identified pain- and emotion-related differentially expressed genes (DEGs) and revealed that numbers of these DEGs displayed a high correlation to neuroinflammation and apoptosis. Consistently, functional analyses showed that the most significant enriched biological processes of the upregulated mRNAs were involved in the immune system process, apoptotic process, defense response, inflammation response, and sensory perception of pain across three regions. Moreover, the comparisons of pain-, anxiety-, and depression-related DEGs among three regions present a particular molecular map among the spinal cord and supraspinal structures and indicate the region-dependent and region-independent alterations of gene expression after nerve injury. Our study provides a resource for gene transcript expression patterns in three distinct pain-related regions after peripheral nerve injury. Our findings suggest that neuroinflammation and apoptosis are important pathogenic mechanisms underlying neuropathic pain and that some DEGs might be promising therapeutic targets.

Keywords: SNL (spinal nerve ligation), neuropathic pain, emotion disorder, spinal cord (SC), anterior cingulate cortex (ACC), amygdala (AMY), RNA sequencing, differentialli expressed genes (DEGs)

INTRODUCTION

Neuropathic pain characterized by a broad range of sensory, cognitive, and emotional dysfunction is a complex and debilitating public health problem that affects about 7–10% of the gross population worldwide (van Hecke et al., 2014; Bushnell et al., 2015; Colloca et al., 2017). Clinical and preclinical investigations have observed clusters of behavioral symptoms including spontaneous pain, evoked nociceptive behaviors, pain aversiveness, anxiety, and depression in neuropathic pain rodents and patients (Seminowicz et al., 2009; LaCroix-Fralish et al., 2011). These abnormal behaviors may involve the changes in the activities of nociceptive neurons and the emergence of the new pathological processes and signaling pathways (von Hehn et al., 2012; Guo et al., 2016). However, current treatments have not yielded satisfactory results. Opioids and non-steroidal anti-inflammatory drugs (NSAID) are considered effective approaches to relieve these symptoms, but the efficacy should be re-appraised because of possible safety concerns (Finnerup et al., 2015; Jones et al., 2018). Therefore, identifying the gene expression profiles and gene interactions in pain-associated regions is essential for understanding the pathogenesis under neuropathic pain and developing novel therapeutic strategies to improve the treatment outcomes (Samuel and Farsides, 2017).

Abnormal changes in neural activity and plasticity arising from tissue or nerve injury contribute to pain hypersensitivity (von Hehn et al., 2012). The spinal cord is responsible for receiving information from nociceptors and projecting to the brain and plays a major role in integrating and modulating nociceptive signals (Kuner, 2010). Studies have reported that brain regions anterior cingulate cortex (ACC) and amygdala (AMY) are important areas in pain sensation and involved in the interpretation and assessment of the affective and emotional components of pain (Gao et al., 2004; Phelps and LeDoux, 2005; Cao et al., 2009; Navratilova et al., 2015; Neugebauer, 2015). The lesion of ACC and AMY was documented to inhibit the conditioned place aversion of formalin (Gao et al., 2004; Cao et al., 2009). Increasing evidence indicates that cellular and molecular adaptations within these two regions appear under chronic stress and chronic pain conditions (Simons et al., 2014; Ji et al., 2017; Sellmeijer et al., 2018; Navratilova et al., 2019). However, gene expression patterns in these two areas after nerve injury have not been examined. Moreover, previous studies identified a large amount of differentially expressed genes (DEGs) using gene microarrays and RNA sequencing in neuropathic pain (Jiang et al., 2015; Wu et al., 2016; Descalzi et al., 2017; Zhou et al., 2017), but these studies did not provide a full comparison among distinct pain-associated regions as most of them focused on only one region. Thus, it is imperative to have a comprehensive understanding by sequencing and comparing the gene expression patterns in different pain-associated regions.

To this end, in the present study, we carried out a more thorough analysis of gene expression alterations after nerve injury by examining the DEGs in the spinal cord, ACC, and AMY. A mouse model of L4 spinal nerve ligation (SNL) and the next-generation RNA sequencing with a higher sequencing depth were conducted. Our results revealed the unique transcriptional profiles across three regions responding to peripheral nerve injury and the significant overlapping effects implicating in biological functions and signaling pathways despite some differences. Functional analysis demonstrated that the pain-, anxiety-, and depression-related DEGs were closely associated with neuroinflammation and apoptosis. The conspicuous overlap of pain-, anxiety-, and depression-related DEGs among three regions illustrates some conservative changes in the transcriptome independent of regions. Therefore, our findings may bring some useful information and novel insights into the molecular mechanism that will lead to a new direction for further studies and a potential development of clinical analgesic medications.

METHODS

Animal Preparation

Eight-week-old male C57BL/6 mice (25–28 g) were purchased from the Animal Experiment Center (Zhengzhou, Henan, China) and housed in the central animal facility under a standard 12-h light/12-h dark cycle with food and water *ad libitum*. The mice were kept for at least 7 days before the experiments. The procedures for the care and use of animals were approved by the Animal Care and Use Committee of Zhengzhou University and performed under the guidelines of the International Association for the Study of Pain.

L4 SNL-Induced Neuropathic Pain Model

Mice underwent unilateral L4 SNL (the fourth lumbar L4 spinal nerve) as previously described (Wu et al., 2016; Zhou et al., 2017; Sun et al., 2019). Briefly, animals were anesthetized with isoflurane and the L4 spinal nerve was exposed through the removal of the L5 transverse process. After the exposure and isolation of the L4 spinal nerve, a tight ligation with 7–0 silk thread was made and the nerve was transected distal to the ligature. The surgical procedure for the sham group was identical to that of the SNL group, except that the spinal nerves were not transected or ligated.

Behavioral Testing

Animals were habituated to the testing room with a stable temperature for at least 1 day before behavioral measurements.

Von Frey Filament Testing

Mice were put in individual Plexiglas chambers elevated on the mental mesh screen, and 30 min was allowed for adapting to the environment before the testing. The calibrated von Frey filaments (0.07 g and 0.4 g) were used to stimulate the plantar surface of each hind paw for 1 second. Each application represented one trial, and 10 trials were performed for each hind paw. The times were recorded when the animal exhibited a response (withdrawal, flicking, flinching, or licking) to the stimulation for each set of 10 trials. The paw withdrawal frequencies in 10 trials were

calculated to evaluate the mechanical sensitivity (Sun et al., 2019; Li Y. et al., 2020).

Hargreaves Assay

Animals were placed in individual Plexiglas chambers on a glass plate and acclimated for 30 min before the testing. The light beam in a Model 336 Analgesia Meter (UGO BASILE S.R.L., Italy) was applied. The radiant heat stimulus was turned off automatically when the animal displayed paw withdrew. The duration between the light application and paw withdrawing was considered as the paw withdrawal latency (PWL). The test for each paw was repeated three to five times at 5-min intervals. A cutoff of 20 s was set to avoid tissue damage (Sun et al., 2019; Li Y. et al., 2020).

Cold Plate Assay

Animals were placed in a chamber on the cold plate with the temperature at 0°C, which was monitored continuously by a thermometer. The duration between the placement and the first sign of mouse jumping and/or flinching was recorded as the paw withdrawal latency (PWL) to noxious cold stimuli. Each test was repeated three times at 10-min intervals for the ipsilateral hind paw. The cutoff of 20 s was set to avoid tissue damage (Sun et al., 2019; Li Y. et al., 2020).

Conditioned Place Aversion (CPA) Testing

The CPA test was carried out as previously described with minor modifications (Guo et al., 2016; Wu et al., 2019). Briefly, the CPA apparatus consisted of two Plexiglas chambers (15 cm × 15 cm × 15 cm) elevated on the mental mesh screen with a removable board in the middle (15 cm × 15 cm). The experimental process included three distinct sessions: a preconditioning session, a conditioning session, and a postconditioning (testing) session with a duration of 10 min in each session. Before the testing, animals experienced the habituation to the apparatus for at least three consecutive days. Six days post-surgery, animals were allowed to freely explore two chambers for 10 min in the preconditioning session. The amount of the time spent in each chamber was recorded. The mice with a strong initial bias (time spent in one chamber > 500 s) were excluded from the study. In the conditioning session, mice were trained in chambers paired with two von Frey filaments. Low-force (0.07 g) von Frey filament was paired with the non-preferred chamber, in which animals spent less time at the preconditioning session. Medium force (0.4 g) was paired with the preferred chamber in which animals stayed longer at the preconditioning session. During the 10-min training, von Frey filaments were used to stimulate ipsilateral hind paw of sham or SNL mice in the corresponding chamber for 5 min with 10-s intervals. Finally, in the postconditioning (testing) session, mice were allowed free access to both chambers. The duration of time that each mouse spent in each chamber was then recorded for 10 min. Results were presented as "Time in chamber" and "CPA score" that was calculated by the time recorded in pre-condition minus the time recorded in the post-condition.

Tissue Collection and RNA Extraction

Briefly, two groups of mice (SNL and Sham) with three biological replicates were used. Unilateral punches were taken from the SC, ACC, and AMY, respectively. The punches per region were pooled from three mice per sample. A total of nine animals per treatment group were needed. Total RNA was extracted using the miRNeasy kit with on-column digestion of genomic DNA (QIAGEN, Valencia, CA, United States) according to the manufacturer's instructions. RNA was purified with RNeasy Micro Kit 50 (cat. 74004, Qiagen), and the concentration was measured using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Sample quality was evaluated with the ratios of A260/280 (1.97∼2.08) and RNA integrity numbers (RIN, 7.5∼8.4) as demonstrated by An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

RNA Sequencing

The total RNA (1.0 μg/sample) was subjected to rRNA depletion by Ribo-Zero rRNA Removal (Human/Mouse/Rat) Kit (Illumina, San Diego, CA, United States). Strand-specific RNA libraries were prepared using TruSeq Stranded Total RNA Sample Preparation Kit (Illumina) without poly-A selection. All assays were performed according to the manufacturer's instructions. RNA-seq was performed on the Illumina Nova6000 plate High Output Model (Illumina, San Diego, United States) (Hrdlickova et al., 2017), in a 2 × 150-bp paired-end configuration, with a total of more than 2,666 M reads per lane (at least 50 M reads per sample).

Bioinformatics Analysis

The samples from the SC, ACC, and AMY were subjected to multiplexing, sequencing, and differential gene expression analysis such as transcript expression analysis and ncRNA expression analysis. Briefly, Trimmomatic 0.32 was used to trim the sequences (minimal length 50 base pairs, leading and trailing Phred Q 30) for the quality first. The resulting sequencing data were then mapped to the musculus genome sequence version GRCm38.72 downloaded from ENSEMBLE. Gene hit counts and reads per kilobase per million (RPKM) were calculated for each gene to determine the expression levels within the CLCbio software environment (CLC Genomics Workbench 7.0.2, CLC genomics Server). Mapped reads were visualized on the UCSC browser using bigwig files converted from bam files. The significant differentially expressed (DE) mRNAs were defined using a cutoff of $P < 0.05$ and fold change ≥ 1.74 ($\log2(\pm 0.8)$) to include more DEGs for the subsequent analyses, such as Go term and KEGG pathway analysis, to get more useful information, especially for the functional analysis and comparisons among three distinct regions. The heatmaps were generated via OmicShare ([1]GENE *DENOVO*). The function of DE mRNAs was analyzed using the downloaded Gene Cards

[1]http://www.omicshare.com/tools

database[2] and Comparative Toxicogenomics Database[3]. The DE mRNAs in the SC were mapped to pain-related genes (refer to pain/itch/touch/thermal/chemical related genes), and the DEGs in the ACC and AMY were aligned to pain- and emotion-related genes (refer to anxiety/depression-related genes). Moreover, the DEGs were compared with the genes related to neuroinflammation (inflammation and immunity) and apoptosis. A Venn diagram was also employed for the DEG comparisons[4].

Quantitative Real-Time RT-PCR

The RNA-sequence results were verified by q-RT-PCR. Total RNA was extracted from tissues as described above, treated using DNase I (New England Biolabs, Ipswich, MA, United States), and finally reversely transcribed with the Revert Aid First Strand cDNA Synthesis Kit (Thermo) according to the manufacturer's instructions either oligo (dT) primers or specific RT primers. A template (2 μL) was used for the amplification by real-time PCR with primers as shown in **Table 1** (Sangon Biotech, Shanghai, China). Each sample was run in triplicate in a 20-μL volume for the reaction containing 250 nM forward and reverse primers, 10 μL Thermo Scientific Maxima SYBR Green qPCR Master Mix (2×; Thermo Scientific Maxima SYBR Green qPCR Master Mix, Rox solution provided), and 20 ng total cDNA. Reactions were implemented in a 7500 Fast Real-Time PCR Detection System (Applied Biosystems, United States). The cycle parameters were set as follows: an initial 3-min incubation at 95°C, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Ratios of ipsilateral-side mRNA levels to contralateral-side mRNA levels were calculated using the Δ Ct method ($2^{-\Delta\Delta Ct}$). All data were normalized to *Tuba1α* (Wu et al., 2016), which was identified as stable in mice after nerve injury.

[2]https://www.genecards.org/

[3]http://ctdbase.org/

[4]https://bioinfogp.cnb.csic.es/tools/venny/index.html

TABLE 1 | Primers for RT-qPCR.

Primer names	Sequences
Tuba1a-F	GTG CAT CTC CAT CCA TGT TG
Tuba1a-R	GTG GGT TCC AGG TCT ACG AA
Cmklr1-F	TGC ATG AAC CCC ATT CTG TA
Cmklr1-R	TGG TGA AGC TCC TGT GAC TG
Adrb3-F	ACA GGA ATG CCA CTC CAA TC
Adrb3-R	TTA GCC ACA ACG AAC ACT CG
P2ry12-F	CCT GGG GTT GAT AAC CAT TG
P2ry12-R	AAC ATG AAG GCC CAG ATG AC
Kcnma1-F	CCC AAT AGA ATC CTG CCA GA
Kcnma1-R	ATC GTT GGC TGC AAT AAA CC
Kcnk18-F	AGG AAG CCA TCC CTC AGA TT
Kcnk18-R	CAG GAG TTG CTC CTC TCC AC
Mir9-3hg-F	CAC ATG CCT AGA CAG GAG CA
Mir9-3hg-R	ACT ATC CAG CCA GTG GGA TG
Miat-F	AAA CCT GAG TCC TGG TGT GG
Miat-R	AAA AAC AGG TGG CCA AAG TG
Pantr1-F	GGA GAG GGA CAG AGT GCC TA
Pantr1-R	AAC CCC TGG ATA GGA CCA AC

RT-qPCR, reversed transcriptase quantitative polymerase chain reaction; F, forward; R, reverse.

Functional Enrichment Analysis of Differentially Expressed Genes (DEGs)

For the function analysis, about 2,230, 1,689, and 1,812 DE mRNAs ($P < 0.05$, fold change ≥ 1.74), respectively, from the SC, ACC, and AMY were categorized using the Kyoto Encyclopedia of Genes and Genomes (KEGG pathway analysis) and Gene ontology analysis by the database for Annotation, Visualization and Integrated Discovery (DAVID[5]) (Li M. et al., 2020). Likewise, GO Annotations and KEGG Pathways Analysis were applied to predict the role of DE lncRNAs through their target mRNAs. The genetic regulatory networks were clarified by forming hierarchical categories according to the BP, MF, and CC aspects[6] (Zhou et al., 2017). The significant pathway enrichments of differentially expressed lncRNAs were predicted by the Pathway Analysis[7] (Zhou et al., 2017).

Protein–Protein Interaction Network Construction

To further analyze and elucidate the functional connection between the differentially expressed encoding genes, the interaction among the significant DEGs in three regions was predicted by the STRING database (version: 11.0)[8]. The top 50 DEGs with the highest correlation degree were screened out to establish the network in the Cytoscape program (version: 3.6.0[9]) (Liu et al., 2019). The connection degree of each node was calculated through the cityscape plugin. The node size was defined by the connection degree. Red and blue colors represented the up- and downregulated genes, respectively.

Co-expression Network Construction

LncRNA–mRNA co-expression networks were established based on the correlation between DE lncRNAs and their neighboring, overlapping, or distant mRNAs in the genome. DEGs with the Pearson correlation coefficients (PCC) > 0.95 or < –0.95 between lncRNAs and mRNAs (FDR < 0.05) were picked out to draw networks by the Cytoscape program (Dong et al., 2014).

Statistical Analysis

All data were collected randomly and expressed as mean ± SEM. The data were statistically analyzed with two-tailed, unpaired Student's *t*-test, and one-way and two-way ANOVA with repeated measures. When ANOVA showed a significant difference, pairwise comparisons between means were tested by the *post hoc* Tukey method. Values of $P < 0.05$ were considered statistically significant. The data were analyzed by GraphPad Prism 8.0.

[5]http://david.abcc.ncifcrf.gov/

[6]http://www.geneontology.org

[7]http://www.genome.jp/kegg/

[8]https://string-db.org/cgi/

[9]www.cytoscape.org/

RESULTS

SNL Leads to Nociceptive Hypersensitivities and Pain Aversiveness Behaviors

Consistent with early reports (Jiang et al., 2015; Wu et al., 2016), mice exposed to SNL showed significant mechanical allodynia (**Figures 1A,B**), thermal hyperalgesia (**Figure 1C**), and cold hyperalgesia (**Figure 1D**) as indicated by the increases in paw withdrawal frequencies in response to von Frey filament stimuli and the decreases in paw withdrawal latencies in response to heat and cold stimuli, respectively, on day 7 post-surgery on the ipsilateral side as compared to those on the contralateral side. As expected, no nociceptive hypersensitivities were observed on either side of sham-operated mice (**Figures 1A–D**). Previous studies indicated that peripheral nerve injury led to emotional aversion on day 3 postsurgery (Suzuki et al., 2007; Wu et al., 2019). In line with these studies (Guo et al., 2016; Wu et al., 2019), the time spent in the preferred chamber at initial was sharply reduced in SNL mice on day 7 postsurgery after the repeated stimuli of 0.4 g von Frey filament (**Figure 1E**;

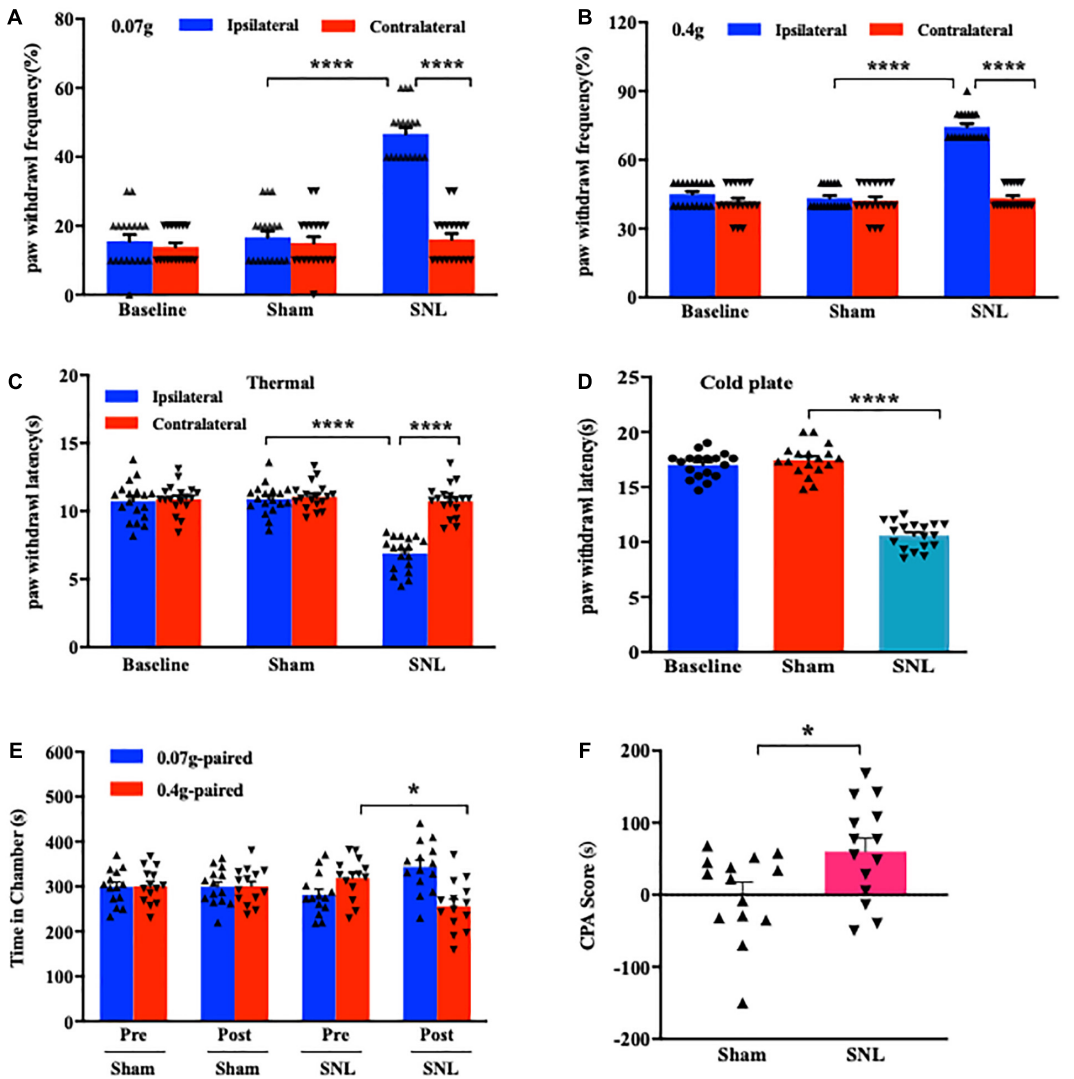

FIGURE 1 | Unilateral L4 spinal nerve ligation (SNL) produced nociceptive hypersensitivities and pain aversiveness in mice. **(A,B)** Paw withdrawal frequencies in response to 0.07 g **(A)** and 0.4 g **(B)** von Frey filament stimuli on the ipsilateral and contralateral sides on day 7 following SNL or Sham surgery. n = 18/group. ****$P < 0.0001$ versus the corresponding sham group or the corresponding contralateral side by two-way ANOVA with repeated measures followed by *post hoc* Tukey test. **(C,D)** Paw withdrawal latencies in response to thermal **(C)** and cold **(D)** stimuli on the ipsilateral and contralateral sides on day 7 post-SNL or -Sham surgery. n = 18 mice/group for thermal test and 14 mice/group for the cold test. ****$P < 0.0001$ versus the corresponding sham group or the corresponding contralateral side by one-way **(D)** or two-way **(C)** ANOVA with repeated measures followed by *post hoc* Tukey test. **(E,F)** Time spent in the corresponding chamber paired with 0.07 g or 0.4 g von Frey filament stimuli **(E)** and CPA score **(F)** in the sham-operated and SNL-operated groups. n = 14 mice/group. Pre: Precondition. Post: Postcondition. N.S: not significant, $P > 0.99$. *$P < 0.05$ versus the corresponding precondition **(E)** or sham group **(F)** by two-way ANOVA with repeated measures followed by *post hoc* Tukey test **(E)** or two-tailed unpaired Student's *t*-test **(F)**.

$^{*}P < 0.05$), demonstrating that SNL mice exhibited emotional aversion. As expected, sham-operated animals did not show any differences when they received the same training (**Figure 1E**; $P > 0.99$). The difference in the CPA score between SNL- and sham-operated groups was statistically significant (**Figure 1F**). Taken together, SNL mice exhibited well-established nociceptive hypersensitivities and emotional aversiveness.

RNA-Seq and Genome-Wide Read Mapping in the SC, ACC, and AMY After SNL

More than 50 million (M) reads in each group per region (SC: 55.88 M–95.96 M in sham and 80.78 M–86.10 M in SNL; ACC: 90.74 M-102.62 M in sham and 68.88 M–86.05 M in SNL; AMY: 98.44 M–111.45 M in sham; and 93.65 M–126.80 M in SNL) were achieved. After the trimmed reads were mapped to the reference mouse genome from ENSEMBLE (GRCm38.90), mapped reads were sorted through as exonic, intronic, and intergenic. The proportion of the reads within each category in sham and SNL groups from the SC, ACC, and AMY were illustrated in **Supplementary Figure 1A**. As expected, many reads were aligned to exonic regions in both groups followed by a considerable proportion of reads mapped to intronic regions (**Supplementary Figure 1A**). The reads mapped to intergenic regions accounted for a small percentage in both sham and SNL groups among the three regions (**Supplementary Figure 1A**). Furthermore, **Supplementary Figures 1B,D** illustrated a robust elevation in the level of reads mapped to the exonic region and a remarkable reduction in the proportion of reads aligning to intronic regions ($^{*}P < 0.05$; $^{**}P < 0.01$) in the SC and AMY, implicating the changes in the functional proteins and signaling pathways after SNL. However, no significant changes were observed in the ACC (**Supplementary Figure 1C**).

We then analyzed the expression profiles of the DEGs in the SC, ACC, and AMY. Six days after SNL, approximately 38,584, 38,045, and 38,727 genes out of a total of 102,711, 101,218, and 102,589 transcripts, respectively, were identified in the SC, ACC, and AMY. The numbers of the changed genes in three regions were quite similar. In agreement with the changes in the injured DRGs (Wu et al., 2016), the largest transcriptional changes were observed in protein-coding RNAs (49%), followed by other non-coding RNAs (43–44%) and lncRNAs (7–8%) in three regions on day 7 after SNL (**Supplementary Figures 1E–G**).

Altered Expression Profiles of mRNAs and lncRNAs in the SC, ACC, and AMY After SNL

The robust changes in gene expression of mRNAs and lncRNAs within the spinal cord, ACC, and AMY were observed after nerve injury. About 2,230 (1,616 upregulated, 614 downregulated), 1,689 (1,022 upregulated, 667 downregulated), and 1,812 (1,256 upregulated, 556 downregulated) mRNAs were significantly changed in the SC, ACC, and AMY, respectively (**Supplementary Material 1**). Besides, approximately 196 (30 upregulated, 136 downregulated), 94 (52 upregulated, 42 downregulated), and 131(50 upregulated, 86 downregulated) lncRNAs were significantly altered in the SC, ACC, and AMY, respectively (**Supplementary Material 2**). The clustered heatmaps of DE mRNAs (**Figure 2A**) and DE LncRNAs (**Figure 2B**) revealed distinct gene expression patterns across three regions after SNL.

Venn diagram was applied to further determine whether these DEGs showed co-expression patterns across three regions. The analyses characterized the co-expression genes by comparing up- and downregulated mRNAs and lncRNAs in the SC, ACC, and AMY. We found the co-regulation of DE mRNAs (**Figures 2C,D**) and DE lncRNAs (**Figures 2E,F**) in all three regions, but the robust co-expression patterns were seen in the co-upregulated mRNAs (**Figure 2C**), and the co-downregulated lncRNAs (**Figure 2F**), especially in the SC and AMY (**Figures 2C,F**). The detailed co-expressed DE mRNAs and DE lncRNAs were listed in **Supplementary Materials 1, 2**, respectively.

Highest Differentially Expressed G Protein-Coupled Receptor mRNAs, Ion Channel mRNAs, and lncRNAs in the SC, ACC, and AMY After SNL

G protein-coupled receptors (GPCRs), ion channels, and lncRNAs are critical in the transmission and modulation of nociceptive information (Zhao et al., 2013; Campbell and Smrcka, 2018; Klein and Oaklander, 2018). Besides 7–8% of the whole transcriptome that are lncRNAs, about 617, 552, and 598 DEGs were identified as GPCR mRNAs and 257, 253, and 250 DEGs were identified as ion channel mRNAs in the SC, ACC, and AMY, respectively. The top 15 up- and downregulated DEGs of GPCR, ion channel, and lncRNA across three regions were displayed in the heatmaps (**Figure 3** and **Supplementary Figures 2, 3**). Consistent with previous reports, the levels of the GPCRs P2ry12 (Horváth et al., 2014), Gpr151 (Jiang et al., 2018), Prokr2 (Maftei et al., 2014), Ccr1 and Ccr5 (Eltayeb et al., 2007; Pevida et al., 2014) in the spinal cord (**Figure 3A**); D1 receptor (D1R) (Darvish-Ghane et al., 2020) and Cmklr1 (Guo et al., 2012; Doyle et al., 2014) in the ACC (**Supplementary Figure 2A**); and GPCRs C5ar2 (Carpanini et al., 2019) and Cckbr (Bowers and Ressler, 2015) in the AMY (**Supplementary Figure 3A**) were remarkably increased on day 7 after SNL. In contrast, the amounts of GPCR histamine receptor H1R (Huang et al., 2017) and G protein-coupled receptor 35 (Gpr35) (Cosi et al., 2011) in the spinal cord (**Figure 3A**), Grm5 (Ramos-Prats et al., 2019) in the ACC (**Supplementary Figure 2A**), and Gpr35 (Savitz et al., 2015) and Lpar1 (Pedraza et al., 2014; González de San Román et al., 2019) in the AMY (**Supplementary Figure 3A**) were dramatically decreased after SNL. For the ion channels, we observed the observably elevated expression of Cacna2d2 (Yu et al., 2019), Orai1 (Dou et al., 2018), Aqp9 (Wu et al., 2020), and Trpc6 (Jin et al., 2017; Wang et al., 2020) in the spinal cord (**Figure 3A**), Gria1 (Toyoda et al., 2009) and Cacna1c (Jeon et al., 2010) in the ACC (**Supplementary Figure 2B**), and Cacna1c (Temme and Murphy, 2017), Cacna2d1 (Chen et al., 2018; Young et al., 2016), and Trpc6 (Jin et al., 2017; Wang et al., 2020) in the AMY (**Supplementary Figure 3B**) after SNL. On the contrary, significant reductions were seen in the levels of ion channel transcripts for Kcnq5 (Manville and Abbott, 2018), Cacna1b

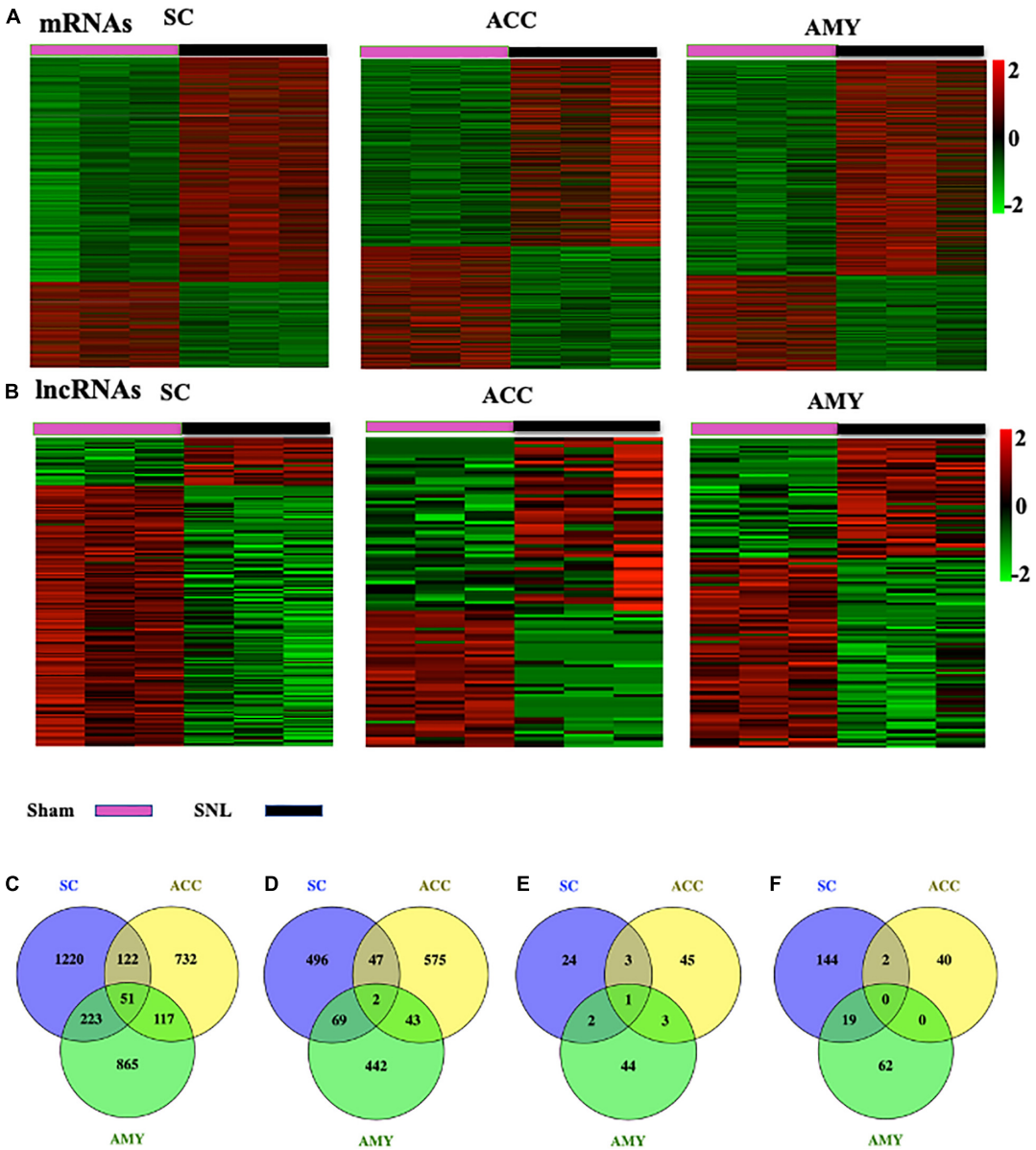

FIGURE 2 | Differentially expressed mRNAs and lncRNAs in the SC, ACC, and AMY after nerve injury. **(A,B)** Heatmaps of significantly differentially expressed mRNAs **(A)** and lncRNAs **(B)** in the SC, ACC, and AMY from mice on day 7 post-SNL or -sham surgery. *n* = 9 mice/group. **(C,D)** Venn diagrams indicate the co-upregulated mRNAs **(C)** and co-downregulated mRNAs **(D)** in the SC, ACC, and AMY on day 7 postsurgery. *n* = 9 mice/group. **(E,F)** Venn diagrams indicate the co-upregulated lncRNAs **(E)** and co-downregulated lncRNAs **(F)** in the SC, ACC, and AMY on day 7 postsurgery. *n* = 9 mice/group.

(Stevens et al., 2019), and Kcnj6 (Zheng et al., 2015) in the spinal cord (**Figure 3B**), Gabrb3 (Tripp et al., 2012), Gabra1 (Chandley et al., 2015), and Grik2 (Chandley et al., 2015) in the ACC (**Supplementary Figure 2B**), as well as the downregulation of the ion channels such as Scn1a, Ano1, Cacna1h (Gangarossa et al., 2014), Cacna1d (McKinney et al., 2009), and Gabra1 (Guilloux et al., 2012) in the AMY (**Supplementary Figure 3B**) on day 7 after SNL. Interestingly, we detected the upregulation of P2ry12 mRNA in the AMY, which was inconsistent with an earlier report, in which the level of P2ry12 mRNA was unaltered in the AMY post-nerve injury (Barcelon et al., 2019).

Additionally, top 15 up- and downregulated lncRNAs in three regions were shown in heatmaps (**Figure 3** and **Supplementary Figures 2, 3C**). As expected, some DEGs for lncRNAs that were identified in the present study (**Table 2**) have been previously reported to implicate in pain (Li et al., 2018; Tang et al., 2018; Che et al., 2019; Meng et al., 2019; Han et al., 2020; Wen et al., 2020; Wu et al., 2020). Consistently, we detected the increased expression of lncRNA Dancr and H19, and the decreased expression of lncRNA Meg3 in the spinal cord (Li et al., 2018; Tang et al., 2018; Che et al., 2019; Han et al., 2020; Wen et al., 2020). The downregulation of lncRNA Malat1 in the spinal

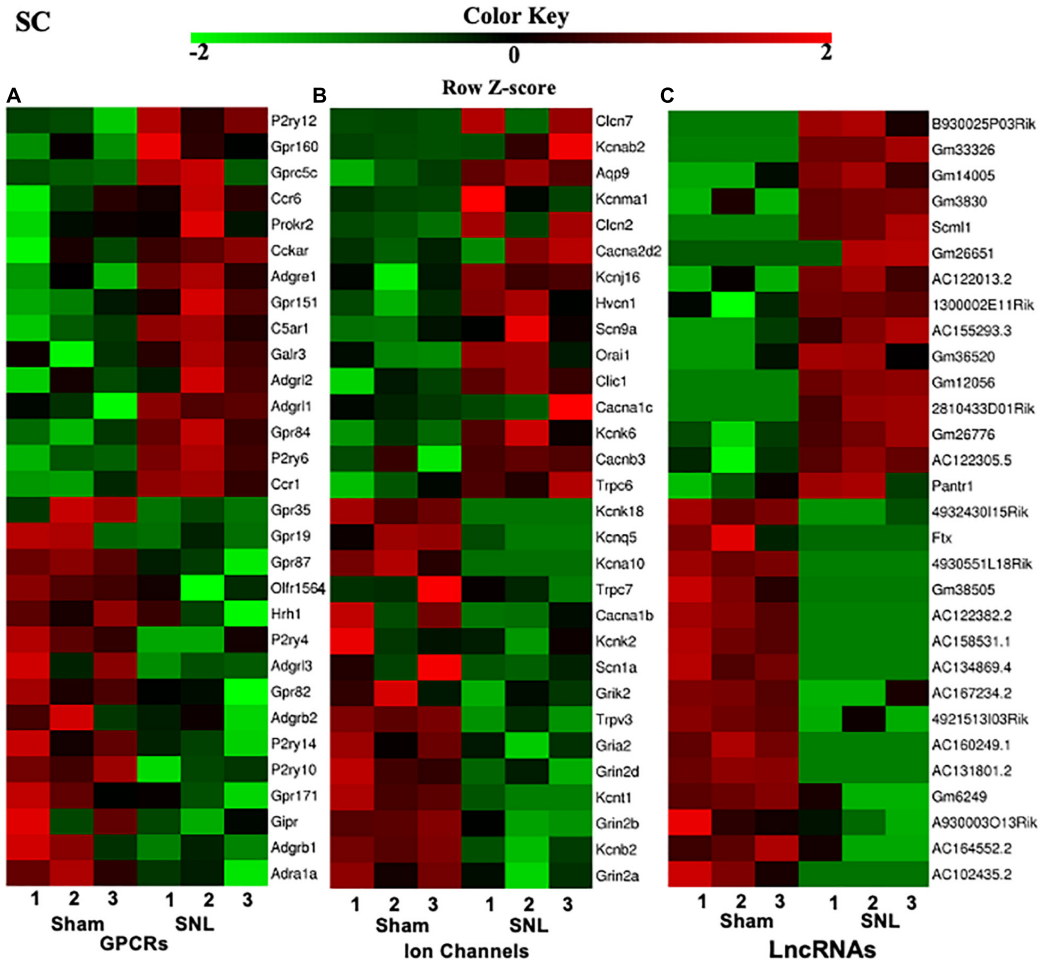

FIGURE 3 | Heatmaps of the representative differentially expressed genes (DEGs) in the spinal cord after nerve injury. **(A-C)**, Top 15 up- and downregulated genes of G protein-coupled receptors **(A)**, ion channels **(B)**, and lncRNAs **(C)** in the fourth lumbar spinal cord on day 7 after SNL. Colors in the heatmaps indicate the Row Z-score among the different datasets. The up- and downregulated genes are colored in red and green, respectively. *n* = 9 mice/group.

cord and the upregulation of lncRNA Meg3 and lncRNA H19 in the ACC were observed on day 7 after SNL, but the functions of these lncRNAs under neuropathic pain conditions need to be further determined (Meng et al., 2019; Wu et al., 2020).

Validation of the DEGs for lncRNAs and mRNAs in the SC, ACC, and AMY

We next conducted a quantitative real-time RT-PCR assay to validate the reliability of RNA sequencing results by analyzing the expression of significant DE lncRNAs and mRNAs on day 7 after SNL in three regions. The expression of three lncRNAs (Pantr1, Mir9-3hg, and Miat), two ion channel mRNAs (Kcnk18 and Kcnma1), and three G protein-coupled receptor mRNAs (P2ry12, Cmklr1, and Adrb3) was measured in the SC (**Figure 4A**), ACC (**Figure 4B**), and AMY (**Figure 4C**), respectively. As expected, the levels of the selected lncRNAs and mRNAs were concomitant with the sequencing results (**Figures 4A–C**). It was noteworthy that the amount of Kcnma1 was elevated in the AMY (**Figure 4C**) but reduced in the ACC (**Figure 4B**).

Functional Enrichment Analysis of the Differentially Expressed Genes After SNL

To explore the functional enrichments of these DEGs, we performed Gene Ontology and KEGG pathway analyses to categorize the up- and downregulated mRNAs based on the distinct processes using the DAVID bioinformatics

TABLE 2 | Spinal nerve ligation-induced differentially expressed lncRNAs that have been previously reported implicated in pain.

Name	Description	Fold change	Ref.
Malat1	Metastasis-associated lung adenocarcinoma	0.68 (SC)	Meng et al., 2019; Wu et al., 2020
Meg3	Maternally expressed 3	0.22(SC); 2.14(ACC)	Li et al., 2018; Che et al., 2019
Dancr	Differentiation antagonizing non-coding RNA	6.34 (n.s) (SC)	Tang et al., 2018
H19	LncRNA H19	2.22(SC); 3.69(ACC)	Han et al., 2020; Wen et al., 2020

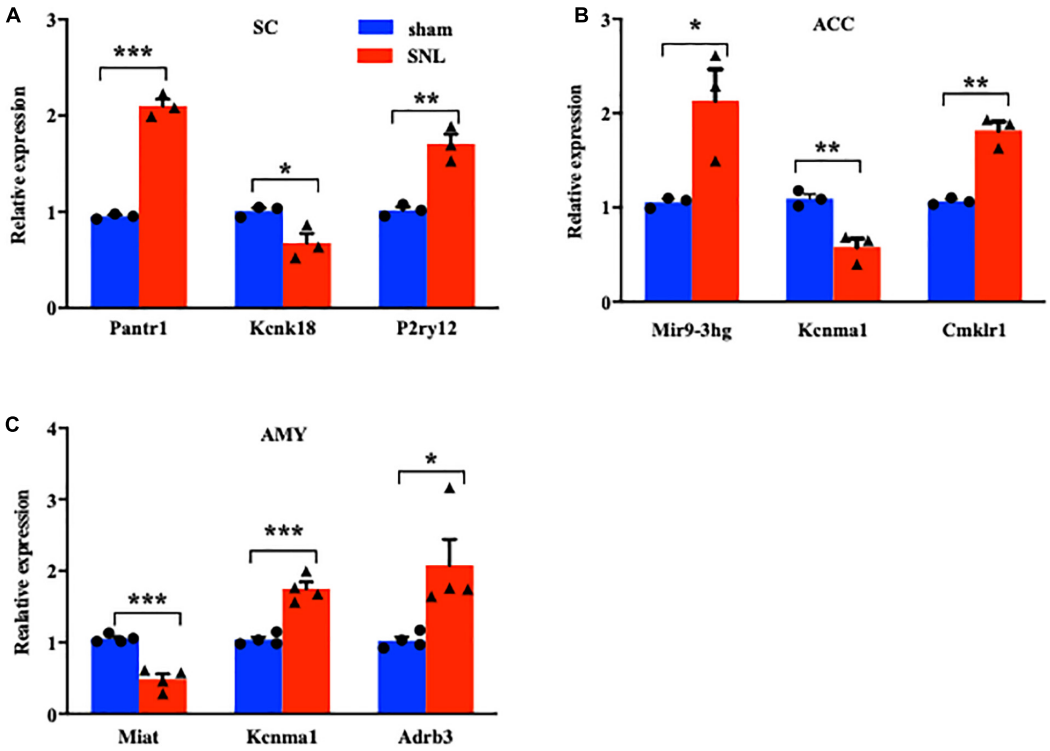

FIGURE 4 | Validations of differentially expressed lncRNAs and mRNAs in the SC, ACC, and AMY after nerve injury. **(A)** Levels of lncRNA Pantr1, G protein-coupled receptor P2ry12, and ion channel Kcnk18 in the spinal cord on day 7 after SNL. $n = 12$ mice/group. $^{*}P < 0.05$; $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the corresponding sham group by two-tailed unpaired Student's *t*-test. **(B)** Amounts of lncRNA Mir9-3hg, G protein-coupled receptor Cmklr1, and ion channel Kcnma1 in the ACC on day 7 after SNL. $n = 12$ mice/group. $^{*}P < 0.05$; $^{**}P < 0.01$, versus the corresponding sham group by two-tailed unpaired Student's *t*-test. **(C)** Levels of lncRNA Miat, G protein-coupled receptor Adrb3, and ion channel Kcnma1 in the AMY on day 7 after SNL. $n = 12$ mice/group. $^{*}P < 0.05$; $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the corresponding sham group by two-tailed unpaired Student's *t*-test.

database. The top 10 analyzed results of biological processes from the up- (red panels on the left) and downregulated (blue panels on the right) mRNAs in three regions were displayed in **Figure 5** and **Supplementary Material 3**. The most significant enriched biological processes of upregulated genes in the spinal cord were immune system process, apoptotic process, innate immune process, inflammatory response, defense response, regulation of RNA transcription, and cell proliferation, while the downregulated genes in the spinal cord were mainly involved in protein phosphorylation, covalent chromatin modification, negative regulation of NF-kappaB transcription factor activity, and cytokine production after nerve injury (**Figure 5A**). The upregulated DEGs in the ACC were highly enriched in transcription, regulation of transcription, signal transduction, locomotory behavior, and sensory perception of pain and G protein-coupled receptor pathways, in contrast to the prominent enrichments in transport, cell differentiation, neuron migration, and positive regulation of synapse assembly for downregulated genes (**Figure 5B**). The upregulated genes in the AMY were related to cell proliferation, apoptotic process, nerve system development, and Histone H4 acetylation besides the enrichments in transcription-related processes, whereas the downregulated genes in the AMY were markedly enriched in regulation of membrane potential, response to wounding, and neuron projection extensive as well as transport processes (**Figure 5C**). For the molecular function enrichments, we observed the striking enrichments in protein binding, DNA binding, and protein homodimerization activity for the upregulated genes and metal ion binding, ATP binding, and transferase activity for downregulated genes in the spinal cord (**Supplementary Material 3**). In the ACC, the upregulated genes were prominently implicated in protein binding, action binding, ion channel binding, and protein kinase binding, while the downregulated genes were involved in protein binding, transcription factor binding, and DNA binding (**Supplementary Material 3**). In the AMY, the upregulated genes were distinctly enriched in protein binding, protein N-terminal binding, and chromatin binding, while the downregulated genes were mainly enriched in lipid binding, endopeptidase inhibitor activity, and protein homodimerization activity (**Supplementary Material 3**). Within the category of "cellular component," the DEGs in three regions were robustly enriched in membrane, cytoplasm, and nucleus (**Supplementary Material 3**).

Pathway analyses showed that most significant pathway enrichments in the spinal cord contained chemokine signaling pathway, tumor necrosis factor (TNF) signaling pathway, and Fc gamma R-mediated phagocytosis for the upregulated genes (red

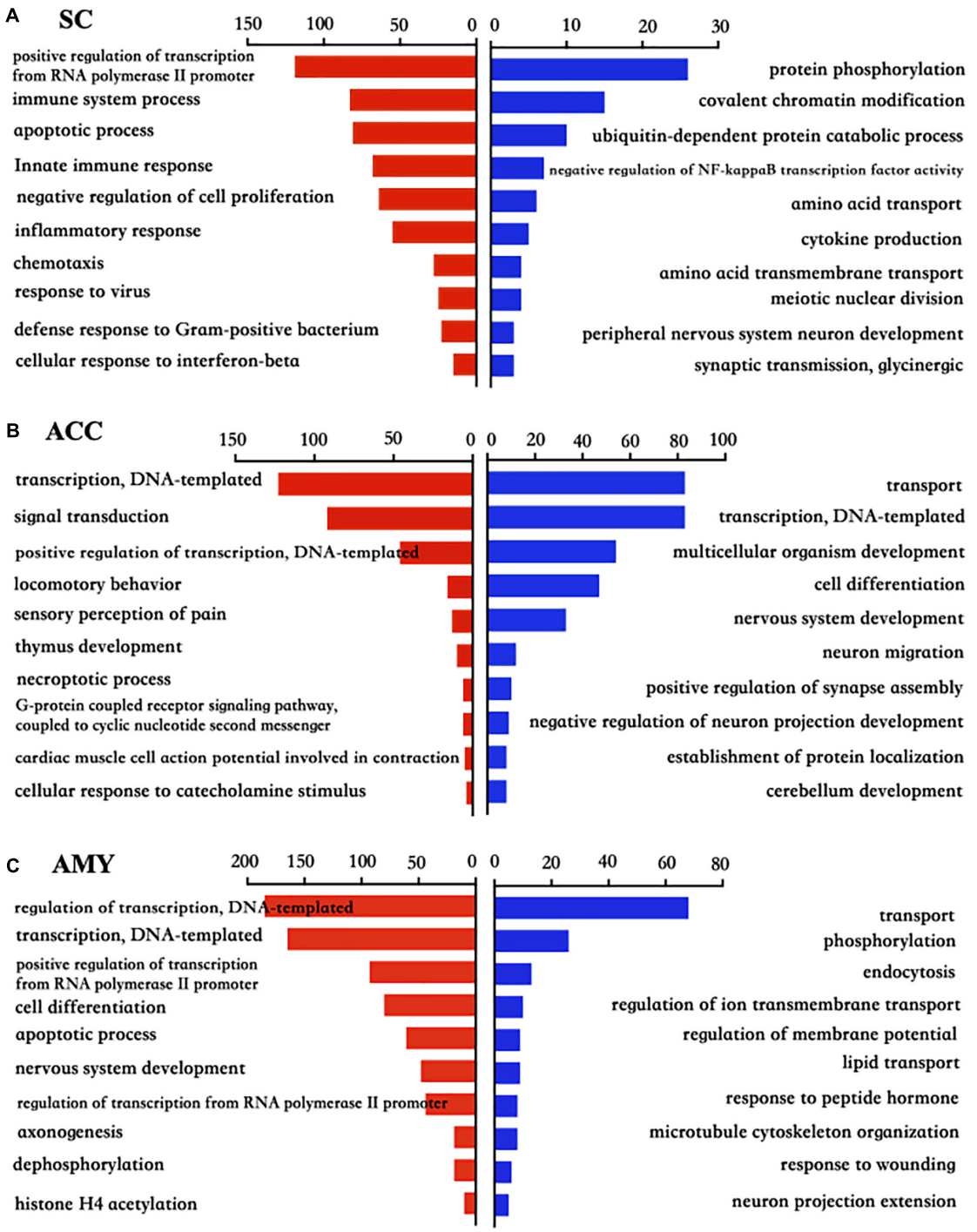

FIGURE 5 | Biological process analysis of the differentially expressed up- and downregulated mRNAs in the SC, ACC, and AMY after nerve injury. **(A–C)** Analysis of the Gene Ontology database showed top 10 biological processes from upregulated mRNAs (red panels on the left) and downregulated mRNAs (blue panels on the right) in the SC **(A)**, ACC **(B)**, and AMY **(C)** on day 7 post-SNL according to the *P*-value. The DAVID database was used to do the GO enrichment analysis. Red and blue bars represent up- and downregulated mRNA enrichments, respectively.

panels on the left) and axon guidance, hippo signaling pathway, and T cell receptor signaling pathway for the downregulated genes (blue panels on the right) (**Figure 6A**). In the ACC, the obvious enrichments were seen in the cAMP signaling pathway, neurotrophin signaling pathway and morphine addiction for the upregulated genes and the insulin signaling pathway, Ras signaling pathway, inflammatory mediator regulation of TRP channels, and type II diabetes mellitus for the downregulated

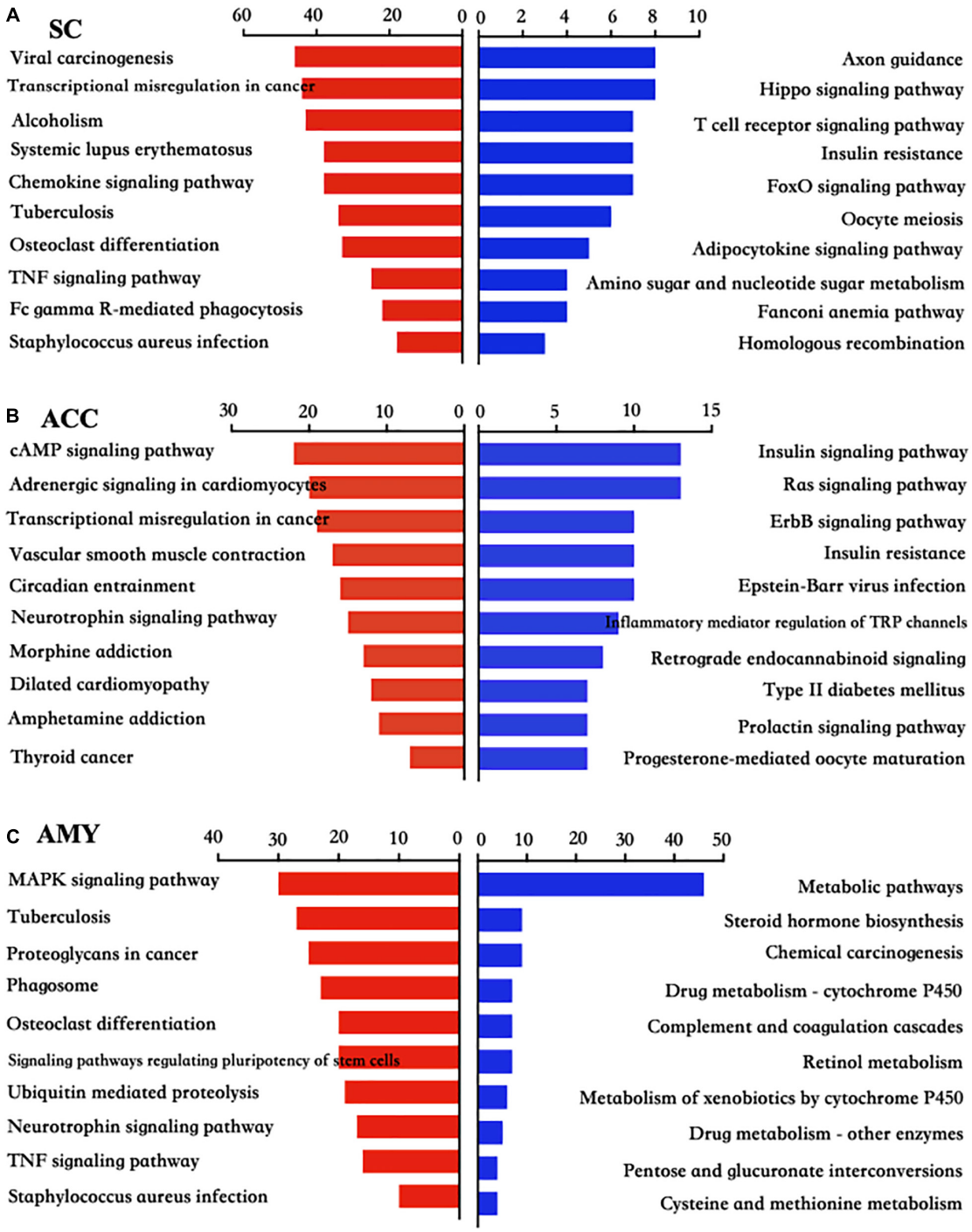

FIGURE 6 | KEGG pathway analysis of the differentially up- and downregulated mRNAs in the SC, ACC, and AMY after nerve injury. **(A–C)** The top 10 enrichments of KEGG pathways from upregulated mRNAs (red panels on the left) and downregulated mRNAs (blue panels on the right) in the SC **(A)**, ACC **(B)**, and AMY **(C)** on day 7 post-SNL according to the *P*-value. The DAVID database was used to do the KEGG pathway analysis. Red and blue bars represent up- and downregulated mRNA enrichments, respectively.

genes (**Figure 6B**). In the AMY, the dramatic enrichments were detected in the MAPK signaling pathway, osteoclast differentiation, TNF signaling pathway for the upregulated genes and the metabolic pathways, chemical carcinogenesis, and complement and coagulation cascades for the downregulated genes (**Figure 6C**). These findings indicated the overlapped function in biological processes and pathways among three regions, which was further verified by the more detailed function

analyses by comparing the function enrichments of the overall DE mRNAs in three regions (**Supplementary Figure 4**).

PPI Network Establishment to Analyze Protein–Protein Interactions in Three Regions After SNL

To gain insight into the functional connection among the DE mRNAs and their potential role in neuropathic pain, a PPI network was conducted using the STRING database. The top 50 protein-coding DEGs with the highest correlation degree in each region were screened out and used to generate the network. As shown in **Figure 7A**, the increased DEGs, such as Itgam, Itgax, Tyrobp, Ptprc, Cd14, Fcgr3, and Cd44, were the crucial molecules among the hub genes in the network of SC, whereas the increased DEGs, such as Trp53, Mapk1, Mapk3, Fn1, Cnb3, Ube2c, Ube2d3, Fbxl19, Cdc34, Keap1, and Lmo7, and the decreased DEGs, such as Atg7, Socs1, Lnx1, Nedd4l, and Uba7, played a major role in the network of ACC (**Figure 7C**). The hub genes in the network of AMY (**Figure 7E**) revealed the vital position of Trp53, Mapk14, Tnf,Icam1, Itgan, Casp3, Myc, and Cd44. In addition, considering

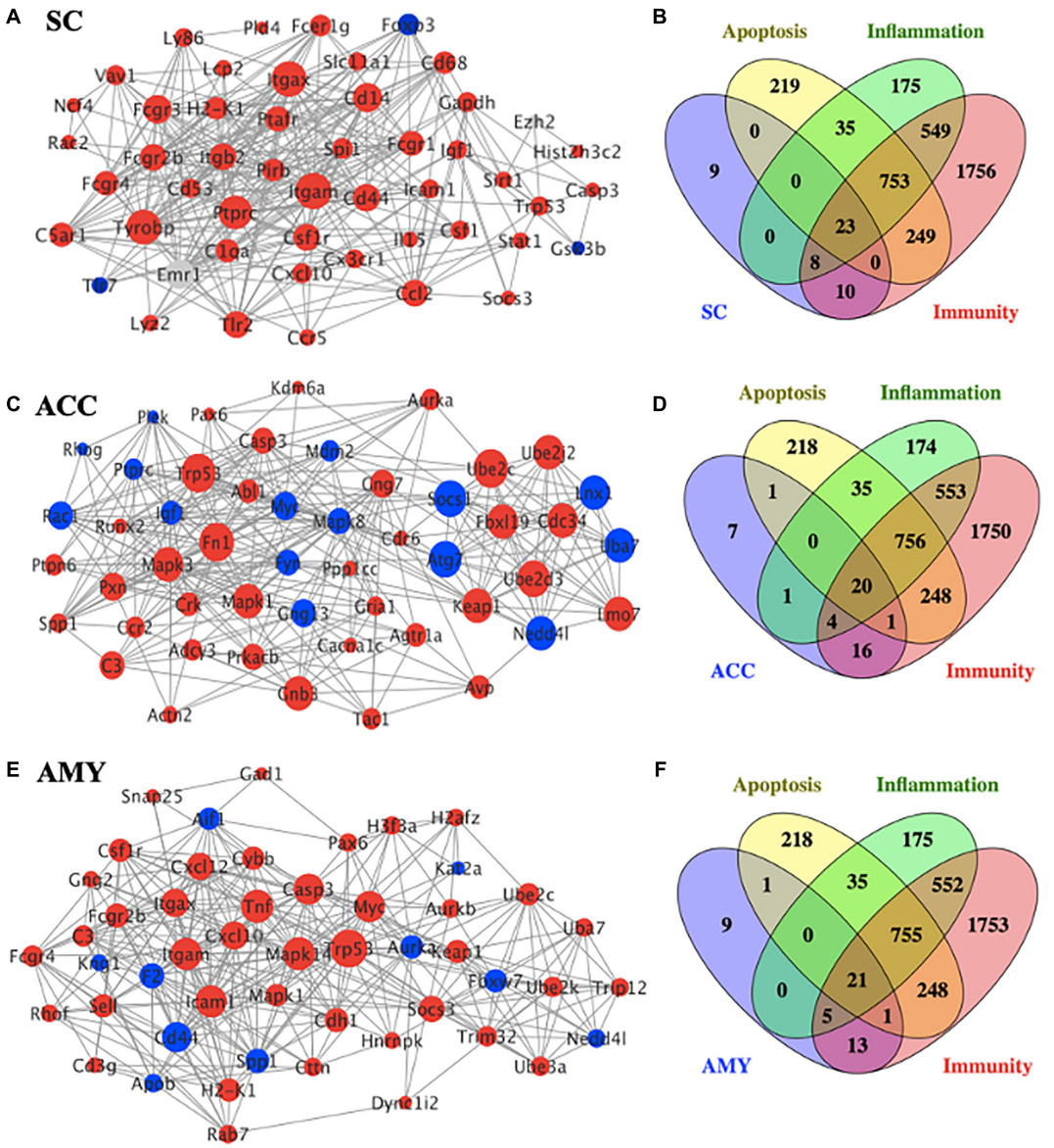

FIGURE 7 | PPI network establishments to analyze protein–protein interactions. (**A**,**C**,**E**) Top 50 differentially expressed genes (DEGs) were picked out based on the connection degree of genes and constructed the network in the SC **(A)**, ACC **(C)**, and AMY **(E)**. The size of the node represents the connection degree that indicates the importance of the gene in the network. Red and blue colors represent the up- and downregulated genes, respectively. (**B**,**D**,**F**) Venn diagrams indicated the number and proportion of the selected top 50 DEGs mapped to apoptosis-, inflammation-, and immunity-related genes in the SC **(B)**, ACC **(D)**, and AMY **(E)**, respectively.

that neuroinflammation and apoptosis participated in many pathological processes including neurological and psychiatric disorders (Chelyshev et al., 2001; Ji et al., 2018; Matsuda et al., 2019), we defined the function of top 50 DEGs in the network of SC, ACC, and AMY by comparing them with a total number of 3,773 genes related to neuroinflammation (inflammation and immunity) and apoptosis (1,543, 3,348, and 1,279 related genes for inflammation, apoptosis, and immunity, respectively). Venn diagrams showed that approximately 82%, 86%, and 82% of DEGs, respectively, from the SC, ACC, and AMY were mapped to neuroinflammation- and apoptosis-related genes (**Figures 7B,D,F**).

Differentially Expressed mRNAs Implicated in Pain, Anxiety, and Depression Disorders Across Three Regions After SNL

We next used the Gene Cards database and CTD database to characterize the DEGs involved in pain and emotional disorders in the SC, ACC, and AMY. Based on the relevance score, about 230 (196 upregulated, 34 downregulated), 157 (100 upregulated, 57 downregulated), and 149 (100 upregulated, 49 downregulated) pain-related DEGs in the SC, ACC, and AMY, respectively, were observed (**Supplementary Figure 5** and **Supplementary Material 4**). The ACC and AMY contained approximately 220 (140 upregulated, 80 downregulated) and 201 (146 upregulated, 55 downregulated) anxiety-related DEGs as well as about 278 (176 upregulated, 102 downregulated) and 287 (207 upregulated, 80 downregulated) depression-related DEGs, respectively (**Supplementary Figure 5** and **Supplementary Material 5**). The top 20 highest up- and downregulated pain-related DEGs were displayed in heatmaps (**Figures 8A–C**).

Pain-, Anxiety-, and Depression-Related DEGs Displayed a High Correlation to Neuroinflammation and Apoptosis in Three Regions After SNL

To further determine the function of DEGs in SNL-induced neuropathic pain pathogenesis, we investigated the correlation between the DEGs implicated in pain, depression, and anxiety disorders and the genes related to neuroinflammation (inflammation and immunity) and apoptosis. About 230 pain-related genes in the SC, 157 pain-related genes, 220 anxiety-related genes, and 278 depression-related genes in the ACC and 149 pain-related genes, 201 anxiety-related genes, and 287 depression-related genes in the AMY were mapped to the neuroinflammation- (inflammation and immunity) and apoptosis-related genes. There were about 51%, 60%, and 71% of pain-related DEGs in the SC; 42%, 52%, and 58% of pain-related DEGs in the ACC; and 47%, 59%, and 68% of pain-related DEGs in the AMY mapping to the datasets of apoptosis, inflammation, and immunity, respectively (**Supplementary Table 1**). Approximately 179, 99, and 110 pain-related overlapping DEGs were seen in three distinct regions, respectively (**Supplementary Table 1**). About 26%, 30%, and 36% of anxiety-related DEGs in the ACC; 31%, 36%, and 49% of anxiety-related DEGs in the AMY; 28%, 30%, and 41% of depression-related DEGs in the ACC; and 27%, 30%, and 44% of depression-related DEGs in the AMY were mapped to the genes associated with apoptosis, inflammation, and immunity, respectively (**Supplementary Table 2**). Furthermore, we found about 88 and 106 overlapping anxiety-related DEGs and 125 and 139 overlapping depression-related DEGs in the ACC and AMY, respectively (**Supplementary Table 2**).

Comparisons of Pain-, Anxiety-, and Depression-Related DEGs Among the SC, ACC, and AMY After SNL

To obtain more information about gene adaptations in response to SNL, we compared pain-related DEGs among three distinct regions in the present study (**Figure 8D**). Strikingly, about 103 overlapping genes were seen among the SC, ACC, and AMY (**Supplementary Table 3**). Of them, we observed the increased expression of C3, Cacna1c, Casp3, Cfh, Crem, Fn1, Men1, Oprl1, Sparc, Trp53, and Txnip and the decreased expression of Scn1a in all three regions. Interestingly, Scn1a was also reported downregulated in injured DRGs (Wu et al., 2016). In addition to the concordant expression patterns, we saw the discrepancy in the expression of the genes such as Alad, Ank1, Aurka, Ccnd3, Hgf, Mtm1, Nrf1, Tcf4, Wnk1, and Tardbp among the SC, ACC, and AMY. Consistent with the earlier findings in the injured DRGs (Wu et al., 2016), the expression of Gabra1 was reduced and the expression of Cacna2d1 was elevated, both in ACC and AMY after SNL. We also detected the upregulation of Acadvl, Acp5, Aifm1, Crh, Crlf1, Cxcl10, Deaf1, Eif4g1, Elane, F13a, Fgfr1, Fgfr3, Fhit, Gnas, Icam1, Il15, Il31ra, Itgam, Lgals1, Socs3, Stim1, Trappc2, Tsc22d3, Ube3a, Vgf, Vim, and Vip and the downregulation of Kif1a and Lipe in both SC and AMY. Finally, there were also many overlapped genes between the SC and ACC including the upregulated expression of Ikzf1, Pdyn, Plaur, Ppp1r1b, Pygl, and Sh2b3 and the downregulated expression of Anxa4, Cacna1b, Hrh1, Nf2, and Sgk1, as well as the inconsistent expression patterns in Arnt, Capn3, Ccr6, Cnbp, Igf1, Mapk8, Nfkb1, Id2, Litaf, Runx2, Tpm1, and Trps1.

Moreover, pain-, anxiety-, and depression-related DEGs in the ACC and AMY were compared (**Figures 8E–G**), A total of 338 pain-, anxiety-, and depression-related DEGs were observed in the ACC and AMY (**Supplementary Table 4**). Besides pain-related DEGs mentioned above, we saw the upregulated expression of Camk2b, Ccnd3, Ccng2, Cyp26b1, Fkbp5, Fgfr3, Gcnt2, Gnas, Gria1, Keap1, Map2, Mapk1, Mapk9, Nr4a2, Nr4a3, Oprk1, Oprl1, Pde4b, Pde7b, Ppfibp1, Psrc1, Tpm1, Tpm3, and Ube2c mRNAs and the downregulated expression of Tcf4, Pln, and Mtm1 mRNAs in both ACC and AMY. Additionally, the genes such as Cryab, Dclk1, Eif5a, Hgf, Magi1, Mef2c, Myc, Nr3c1, Plec Rai1, and Tardbp were downregulated in the ACC but upregulated in the AMY. The genes including Arrdc3, Diablo, Fmo5, Fnbp1, Ggt1, Nos1, Postn, Tcf7l2, and Xiap were significantly elevated in the ACC but reduced in the AMY. These findings suggested some shared pathogenesis mechanisms in

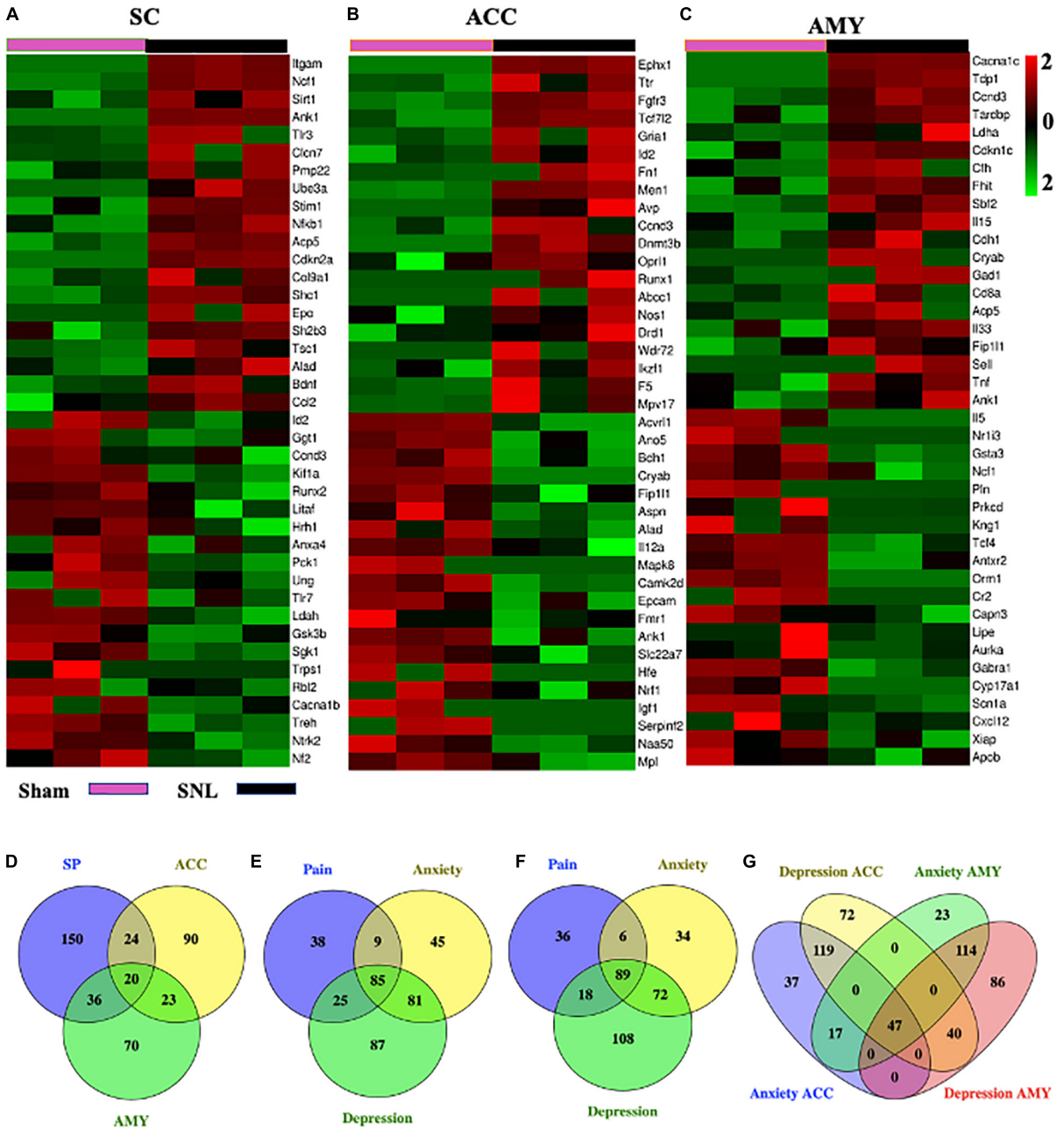

FIGURE 8 | Comparisons of pain-, anxiety-, and depression-related genes in the SC, ACC, and AMY. **(A–C)** Heatmaps of representative top 20 up- and downregulated pain-related DEGs in the SC **(A)**, ACC **(B)**, and AMY **(C)** on day 7 after SNL. **(D)** Venn diagram indicated the number of the overlapped pain-related genes among the SC, ACC, and AMY on day 7 after SNL. Detailed information was displayed in **Supplementary Table 3**. **(E–G)** Venn diagrams indicated the number of overlapped pain-, anxiety-, and depression-related DEGs in the ACC and AMY (detailed information in **Supplementary Table 4**). Colors in the heatmaps indicate the Row Z-score among the different datasets. Up- and downregulated genes are colored in red and green, respectively.

neuropathic pain and emotional disorders, probably providing more evidence and basis for future researches.

Functional Prediction of DE lncRNAs in SNL

We next determined the function of lncRNAs through their related mRNAs by selecting the genes with the absolute value of correlation > 0.95 and the co-localization within 100 kb at the upstream and downstream (**Supplementary Material 6**).

GO enrichment analysis results were graphically displayed in directed acyclic graphs (DAGs), in which the branch represented the relationship of the inclusion that defined the smaller and smaller scales from top to bottom. The top 10 GO enrichments were selected as the master nodes of DAGs.

They were shown together with the GO terms of containment relationships and systematically GO terms. DAGs were plotted from the biological process, molecular function, and cellular component aspects in the SC, ACC, and AMY, respectively (**Figure 9** and **Supplementary Figures 6, 7A-C**). According to the distribution of predicted target genes in the Gene Ontology, the function of DE lncRNAs was clarified and displayed in the form of histograms by the -log (*P*-value) of each GO term. Apparently, the most significant biological process enrichments were synapse assembly, cell–cell adhesion via plasma membrane adhesion molecules, synapse organization, cellular macromolecule metabolic process, nucleic acid metabolic process, and regulation of RNA metabolic process in three regions. The noteworthy cellular component enrichments were seen in intracellular, MHC class I protein complex, nuclear lumen, membrane-enclosed lumen, and intracellular organelle lumen in the SC, ACC, and AMY. The most robust molecular functions were enriched in binding, nucleic acid binding,

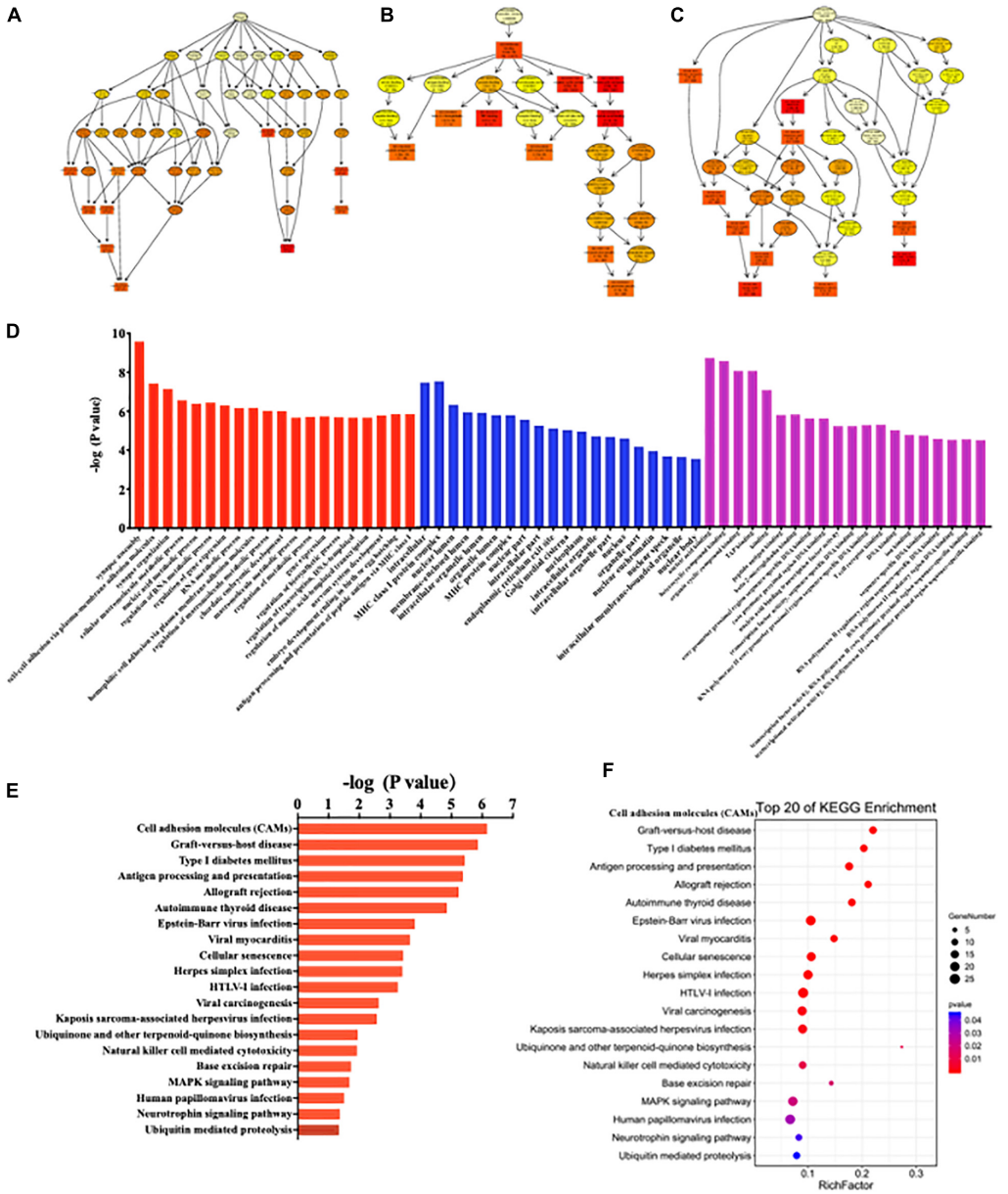

FIGURE 9 | Functional prediction of DE lncRNAs by GO and KEGG analyses in the spinal cord of SNL mice. **(A–C)** Directed acyclic graphs (DAGs) graphically display the significant GO enrichment results with the candidate targeted genes in biological process **(A)**, molecular function **(B)**, and cellular component **(C)**. **(D)** Significant molecular function, biological process, and cellular component enrichment analysis of DE lncRNA-related mRNAs. The enrichment scores (−log10 (*P*-value)) of the GO term were shown in the histogram. **(E,F)** The DE lncRNA-related mRNA-enriched KEGG pathways represented by enrichment scores (−log10 (*P*-value)) **(E)** and the scatterplot showing statistics of pathway enrichment **(F)**, respectively. The color of pathway terms is defined by the enrichment *P*-value.

heterocyclic compound binding, organic cyclic compound binding, and TAP binding among three regions (**Figure 9** and **Supplementary Figures 6, 7D**).

Similarly, the top 20 KEGG enrichments were shown in the histograms by the -log (*P*-value) of each pathway (**Figure 9** and **Supplementary Figures 6, 7E**) and together with the enriched distribution maps (**Figure 9** and **Supplementary Figures 6, 7F**), in which the degree of KEGG enrichment was assessed by the Rich factor, *P*-value, and the number of genes. The enrichment was more significant with the greater rich factor and the larger number of genes but the smaller *P*-value. The most significantly enriched pathways were related to cell adhesion molecules (CAMs), graft-versus-host disease, type 1 diabetes mellitus, antigen processing and presentation, allograft rejection autoimmune thyroid disease, and cellular senescence among three regions after SNL (**Figure 9** and **Supplementary Figures 6, 7F**). Overall, these data demonstrated the overlapped effects in DE lncRNA function among three regions, similar to the patterns of the DE mRNAs.

lncRNA–mRNA Co-expression Network Analysis

To observe the potential interaction between lncRNAs and mRNAs in three regions after SNL, gene co-expression networks were constructed based on the correlation analysis. The networks were established by numbers of DE lncRNAs and the most potential top 50 DE mRNAs targets (PCC $>$ 0.95 or <-0.95, and $FDR < 0.05$) (**Supplementary Material 7**). The cis-acting regulatory networks were constructed with 136 relationships between 88 lncRNAs (61 known, 26 predicted) and top 50 mRNAs in the SC (**Figure 10A**), 137 relationships between 87 lncRNAs (56 known, 31 predicted) and top 50 mRNAs in the ACC (**Figure 10C**), and 137 relationships between 89 lncRNAs (59 known, 30 predicted) and top 50 mRNAs in the AMY (**Figure 10E**). In contrast, the co-expression networks for trans-acting regulation consisted of 80 relationships between 17 lncRNAs (13 known, 4 predicted) and top 50 mRNAs in the SC (**Figure 10B**), 45 relationships between 13 lncRNAs (10 known, 3 predicted) and top 50 mRNAs in the ACC (**Figure 10D**), and 51 relationships between 17 lncRNAs (11 known, 6 predicted) and top 50 mRNAs in the AMY (**Figure 10F**).

DISCUSSION

Neuropathic pain is a somatosensory disorder resulting from nerve injury or diseases affecting the peripheral and central nervous systems (Colloca et al., 2017). With the high incidence and poor management in the clinic, it is a major public health problem. Over the past decades, the potential mechanisms underlying neuropathic pain have been extensively studied. However, the effective treatments are still limited due to the largely unknown molecular mechanisms (Finnerup et al., 2015; Jones et al., 2018). Evidence demonstrates that the alteration in gene expression profiles at different levels of the nervous system plays an important role in the development and maintenance of neuropathic pain. In the present study, we reported gene transcript alterations in pain- and emotion-associated regions in the central nervous system following peripheral nerve injury in mice using the next-generation RNA sequencing assay. Bioinformatics and pathway analyses revealed that particular differentially expressed gene patterns and biological networks in the SC, ACC, and AMY were in correspondence with SNL-induced nociceptive hypersensitivities and pain-related aversion.

In the present study, numerous DEGs belonging to GPCR and ion channel genes were identified. Many of these DEGs have identified function in pain or emotion dysfunction. Ccr1 and Ccr5 were shown to be involved in heat hyperalgesia in mice (Eltayeb et al., 2007; Pevida et al., 2014). The modification of Cmklr1 was shown to implicate depression in the prefrontal cortex and hippocampus responding to chronic restraint stress (CRS) (Guo et al., 2012; Doyle et al., 2014). Genetic knockout of Orai1 nearly eliminated the second phase of formalin-induced pain and attenuated carrageenan-induced pain hypersensitivity and neuronal excitability (Dou et al., 2018). TRPC6 inhibition in the spinal cord blocked the induction of morphine tolerance and hyperalgesia in rats (Jin et al., 2017; Wang et al., 2020). Likewise, we identified the changes in lncRNA expression after SNL. The expression of lncRNA Malat1 was controversial. Following our sequencing result, Meng C et al. demonstrated that the inhibition of spinal Malat1 expression contributed to neuropathic pain after brachial plexus avulsion (Meng et al., 2019). On the contrary, another study reported that the inhibition of spinal Malat1 reduced the incidence of CCI-induced neuropathic pain (Wu et al., 2020). These findings suggest that the molecular mechanism underlying neuropathic pain may vary with different etiologies and courses. LncRNA H19 was upregulated in the injured DRGs and hippocampus neurons following peripheral nerve injury (Han et al., 2020; Wen et al., 2020). Consistently, our sequencing data showed that SNL increased its expression in the spinal cord and ACC. However, whether the increased H19 in these two regions contributes to neuropathic pain needs to be confirmed.

Furthermore, lots of pain-, anxiety-, and depression-related genes were identified in the SC, ACC, and AMY following SNL. Consistent with previous studies, Itgam (CD11b) (Ghasemlou et al., 2015), Tlr3 (Liu et al., 2012), Bdnf (Sapio et al., 2019), and Stim1 (Gao et al., 2016) were significantly elevated, while Kif1a (Wang et al., 2018) and Rbl2 (Chen et al., 2019) were robustly decreased in the spinal cord. However, there was a discrepancy in the expression of Gsk3b. As reported, Gsk3b was downregulated at day 3 and upregulated at day 10 in the spinal cord after partial sciatic nerve ligation (Weng et al., 2014). Unexpectedly, we saw the decreased expression of spinal Gsk3b on day 7 after SNL. These results may imply that spinal Gsk3b expression is time-dependent post-nerve injury. In the ACC and AMY, we observed the upregulation of Drd1 (Darvish-Ghane et al., 2020), Tnf (Akter et al., 2020), and Il33 (Fairlie-Clarke et al., 2018), as well as the increased expression of Runx1 and Cd68 among pain-related DEGs. However, the function of Runx1 and Cd68 in these two regions is still unknown and remains to be investigated.

According to the sequencing data from the injured DRGs (Wu et al., 2016), we observed similar expression changes of some

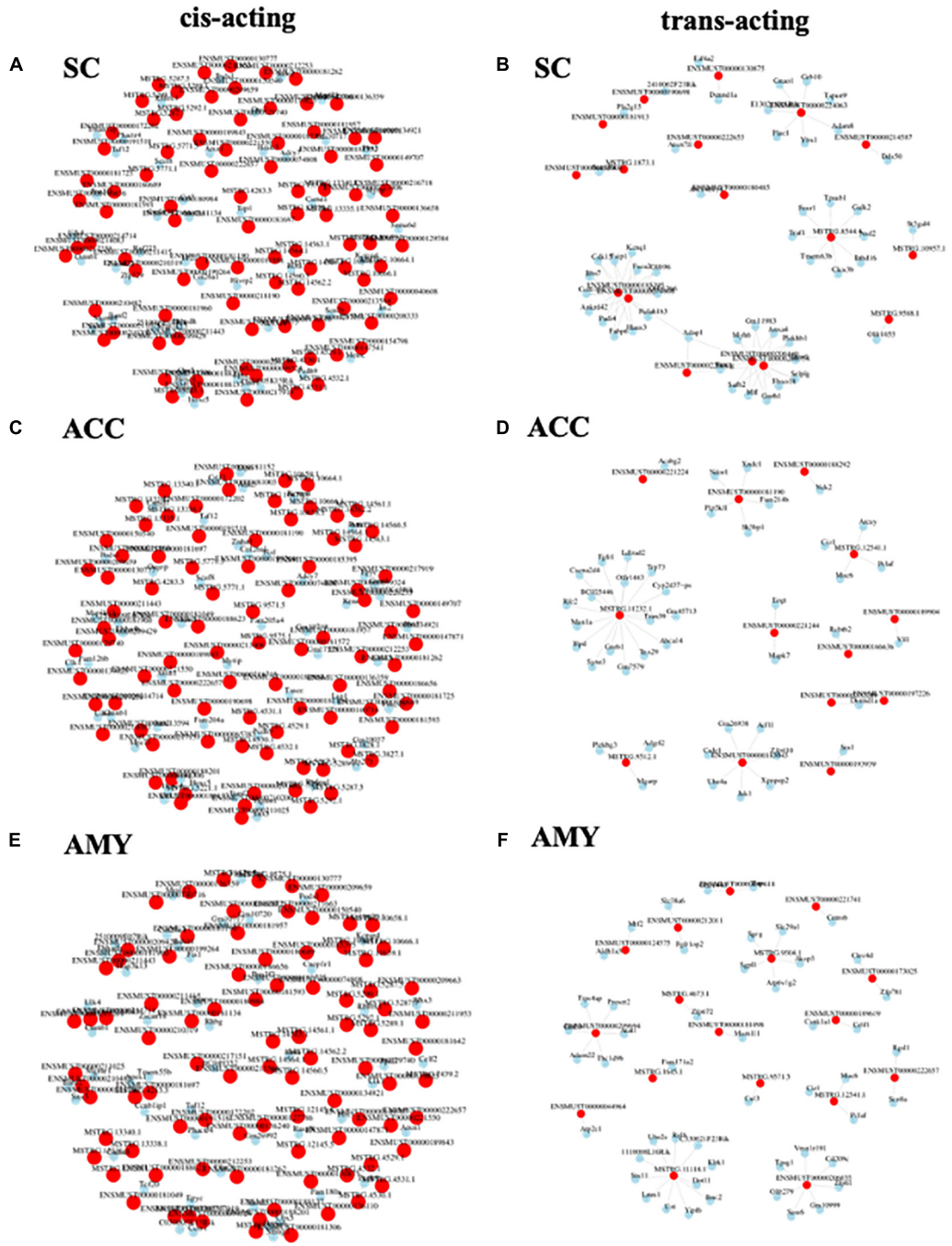

FIGURE 10 | Analysis of lncRNA–mRNA co-expression network in the SC, ACC, and AMY of SNL mice. **(A,B)** Co-expression network of cis-acting regulatory elements **(A)** and trans-acting regulatory elements **(B)** in the SC. **(C,D)** Co-expression network of cis-acting regulatory elements **(C)** and trans-acting regulatory elements **(D)** in the ACC. **(E,F)** The co-expression network of cis-acting regulatory elements **(E)** and trans-acting regulatory elements **(F)** in the AMY. LncRNA–mRNA co-expression network was constructed based on the Pearson correlation coefficients. Pink color nodes represent lncRNA, while blue nodes represent the co-expression mRNAs.

genes such as the upregulation of Atf3, Ccr1, Ccr5, and Gal, and the downregulation of Gria2 in the spinal cord as well as the elevated expression of Cacna2d1 and the reduced expression of Gabra1 in the ACC and AMY after SNL. Besides, we found the consistent expression patterns of some genes such as the upregulation of Atf3, Bdnf, C1qa, C3, Cck, Ccl2, Cd68, Csf1, Cx3cr1, Gch1, Itgam, Ngfr, Pdyn, and Tlr2 in the spinal cord of SNL mice, when compared to the sequencing results in CCI rats (Du et al., 2018; Korczeniewska et al., 2020). These data suggest the common gene expression patterns independent of regions,

models, or species. The ACC and AMY are important brain areas in pain and emotion modulation, but the gene expression profiles in these two regions after nerve injury have not been identified before. We achieved more anxiety- and depression-related DEGs than pain-related DEGs in these two regions, consistent with their significant function in emotion processing. Interestingly, among the anxiety- and depression-related DEGs, we found the upregulation of Bcl3, C3, Gpat3, and Tnf in the AMY as well as the increased expression of Crhr2, Nant, Sar1a, and Tgif1 and the decreased expression of Ier2, Il12a, Nrep, and Tnfrsf25 in the ACC. These alternations were in step with the sequencing results in 12- and 24-month-old mice (Li M. et al., 2020), suggesting some common gene expression alterations in different pathological processes, at least for anxiety- and depression-related genes.

Consistent with previous reports (Jiang et al., 2015; Wu et al., 2016), GO term and KEGG pathway enrichment analyses in three regions showed notable enrichments in apoptotic, inflammation, immunity, cytokine production and defense response, behavior, and sensory perception of pain as well as the enrichments in chemokine signaling pathway, MAPK signaling pathway, TNF signaling pathway, cAMP signaling pathway, Type II diabetes mellitus, and T cell receptor signaling pathway. Consistently, functional analyses observed that large percentages of pain-, anxiety-, and depression-related DEGs were highly related to neuroinflammation and apoptosis that were considered to occupy an important position in pain states (Chelyshev et al., 2001; Ji et al., 2018; Matsuda et al., 2019). Among the overlapped pain-related DEGs, the amounts of Cacna1c, Casp3, C3, and TXNIP were sharply elevated in all three regions following SNL. ACC-conditional deletion of Cav1.2 channels impaired observational fear learning and reduced behavioral pain responses, while neuronal deletion of Cav1.2 led to significant deficits in the extinction of conditioned fear and altered sIPSC and sEPSC activity within the amygdala (Jeon et al., 2010; Temme and Murphy, 2017). Evidence indicated that Casp3 was upregulated in neuropathic pain and that the activation of Casp3 was required in long-term depression (Li et al., 2010; Yang et al., 2018). The deletion of complement C3 was shown to reduce pain-, anxiety-, and depression-like behaviors and to improve learning and spatial memory in aged mice (Shi et al., 2015; Crider et al., 2018). Recent reports suggested that activation of TXNIP/NLRP3 axis was positively associated with pain and emotion disorders and the neuroprotective properties by pharmacological inhibition or genetic deletion of TXNIP following cerebrovascular and neurodegenerative diseases (Nasoohi et al., 2018; Pan et al., 2018). Taken together, the remarkable overlapped DEGs might be the most potential candidates for the researches on pain-, anxiety-, or depression-disorders.

Despite that we reported the unique transcriptome profiles and conducted a series of functional analyses, the present study still has some limitations. Firstly, it should be noted that the SC, ACC, and AMY contain a variety of cell populations including different types of neurons, astrocytes, and microglia cells. However, all bioinformatics analyses presented in this work were obtained from all cell populations. Thus, future studies on the cell-type-specific changes in gene expression following peripheral nerve injury should be performed using single-cell sequencing analysis. Secondly, we used the database of anxiety- and depression-related genes to analyze the DEGs in the ACC and AMY after SNL. However, six days post-SNL, anxiety- and depression-like behaviors were not completely developed even if SNL-induced aversion was detected (Suzuki et al., 2007; Wu et al., 2019). However, the evidence demonstrated that the analyzed anxiety- and depression-related DEGs such as Gria1 (Rivera et al., 2020), Mapk1 (Sierra-Fonseca et al., 2019), Mapk9 (Thomson et al., 2020), and Fkbp5 (Zannas et al., 2019) contributed to anxiety or depression symptoms in rodents. The further study on the gene expression profiles and emotion-related behaviors including anxiety- and depression-like behaviors at the later stage of neuropathic pain should be carried out. Thirdly, investigations on the gender-specific and age-specific pain mechanisms should also be included due to the more frequent prevalence of pain in women and aged patients (Bouhassira et al., 2008). Fourthly, other brain regions such as the medial prefrontal cortex, nucleus accumbens, and periaqueductal gray were reported to participate in pain- and emotion-related behaviors under the conditions of chronic stress and/or chronic pain as well (Bouhassira et al., 2008; Descalzi et al., 2017; Smith et al., 2021). To obtain more valuable information, RNA sequencing analysis at these brain regions needs to be considered in the future. Finally, although the present study demonstrated gene transcript alternations and their functional analyses in the SC, ACC, and AMY, whether these changes contribute to the induction and maintenance of neuropathic pain and whether they can serve as new targets remain to be further determined.

CONCLUSION

In summary, we for the first time provided the unique gene expression profiles of lncRNAs and mRNAs in three pain-related regions and revealed the implication of neuroinflammation and apoptosis in the pathogenesis of neuropathic pain using different bioinformatics analyses. The comparisons of RNA sequencing results provide a more thorough analysis of gene expression alterations in three distinct pain-related regions. Overall, our findings present comprehensive information that may facilitate the discovery of novel analgesic strategies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: National Center for Biotechnology Information (NCBI) BioProject, https://www.ncbi.nlm.nih.gov/bioproject/, PRJNA705299.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of Zhengzhou University.

AUTHOR CONTRIBUTIONS

WZ conceived the project, supervised all the experiments, and edited the manuscript. SS, ML, DW, JC, XR, Y-XT, and WZ assisted with experimental design. SS and ML carried out behavioral tests, surgery, and tissue collection. SS performed the RT-PCR assay and wrote the draft of the manuscript. SS, ML, and DW analyzed the data. Y-XT and WZ edited the manuscript. All authors read and discussed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.634810/full#supplementary-material

Supplementary Figure 1 | Transcriptome profiling in the SC, ACC and AMY in mice after nerve injury. **(A)** The mapped proportions of exonic, intronic, and intergenic reads in the SC, ACC and AMY on day 7 post-SNL or sham surgery. **(B-D)** The proportions of reads that align to exonic, intronic and intergenic regions from the SC **(B)**, ACC **(C)**, and AMY **(D)** on day 7 post-surgery. $n = 3$ biological repeats. $^{*}P < 0.05$; $^{**}P < 0.01$ versus the corresponding sham group by two-tailed unpaired Student's *t*-test. **(E-G)** Distribution of differentially expressed RNAs in SC **(E)**, ACC **(F)**, and AMY **(G)** on day 6 after SNL.

Supplementary Figure 2 | Heatmaps of the representative differentially expressed genes (DEGs) in the ACC after nerve injury. Top 15 up-and down-regulated G protein-coupled receptor mRNAs **(A)**, ion channel mRNAs **(B)**, and lncRNAs **(C)** in the ACC on day 7 after SNL. Colors in the heatmaps indicate the Row Z-score among the different datasets. The up- and down-regulated genes are colored in red and green, respectively.

Supplementary Figure 3 | Heatmaps of the representative differentially expressed genes (DEGs) in the AMY after nerve injury. Top 15 up- and down-regulated G protein-coupled receptor mRNAs **(A)**, ion channel mRNAs **(B)**, and lncRNAs **(C)** in the AMY on day 7 after SNL. Colors in the heatmaps indicate the Row Z-score among the different datasets. The up- and down-regulated genes are colored in red and green, respectively.

Supplementary Figure 4 | The common biological processes and KEGG pathways in the SC, ACC and AMY after nerve injury. **(A-D)** The top 10 common enrichments in biological processes (blue panels on the left) and KEGG pathways (red panels on the right) between SC and ACC **(A)**, SC and AMY **(B)**, or ACC and AMY **(C)**. The top 10 common function enrichments in biological processes (blue panels on the left) and KEGG pathways (red panels on the right) among SC, ACC and AMY **(D)** according to the *P*-value. Blue and red bars represent the biological process and pathway enrichments, respectively.

Supplementary Figure 5 | Heatmaps of the identified pain-, anxiety- and depression-related genes in the SC, ACC and AMY after nerve injury. **(A)** Heatmaps of 157 (100 upregulated and 57 downregulated) pain-, 220 (140 upregulated and 80 downregulated) anxiety- and 278 (176 upregulated and 102 downregulated) depression-related DEGs in the ACC. **(B)** Heatmaps of 149 (100 upregulated and 49 downregulated) pain-, 201 (146 upregulated and 55 downregulated) anxiety- and 287 (207 upregulated and 80 downregulated) depression-related DEGs in the AMY; ***C,*** Heatmaps of 230 (196 upregulated and 34 downregulated) pain-related DEGs in the spinal cord. Colors in the heatmaps indicate the Row Z-score among the different datasets. The up- and down-regulated genes are colored in red and green, respectively.

Supplementary Figure 6 | The functional prediction of DE lncRNAs by GO and KEGG analyses in the ACC from SNL mice. **(A-C)** Directed Acyclic Graphs (DAGs) graphically display the significant GO enrichment results with the candidate targeted genes in biological process **(A)**, molecular function **(B)**, and cellular component **(C)**. **(D)** The significant molecular function, biological process and cellular component enrichment analysis of DE lncRNA-related mRNAs. **(E,F)** The DE lncRNA-related mRNAs enriched KEGG pathways represented by enrichment scores (−log10 (*P*-value)) **(E)** and the scatterplot showing statistics of pathway enrichment **(F)**, respectively. The color of pathway terms is defined by the enrichment *P*-value.

Supplementary Figure 7 | The functional prediction of DE lncRNAs by GO and KEGG analyses in the AMY from SNL mice. **(A-C)** Directed Acyclic Graphs (DAGs) graphically display the significant GO enrichment results with the candidate targeted genes in biological process **(A)**, molecular function **(B)**, and cellular component **(C)**. **(D)** The significant molecular function, biological process and cellular component enrichment analysis of DE lncRNA-related mRNAs. **(E,F)** The DE lncRNA-related mRNAs enriched KEGG pathways represented by enrichment scores (−log10 (*P*-value)) **(E)** and the scatterplot showing statistics of pathway enrichment **(F)**, respectively. The color of pathway terms is defined by the enrichment *P*-value.

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