Behavioural Neurology

Chemical Neuromodulation in Pain and Addictive Disorders

Lead Guest Editor: Andrew Huang
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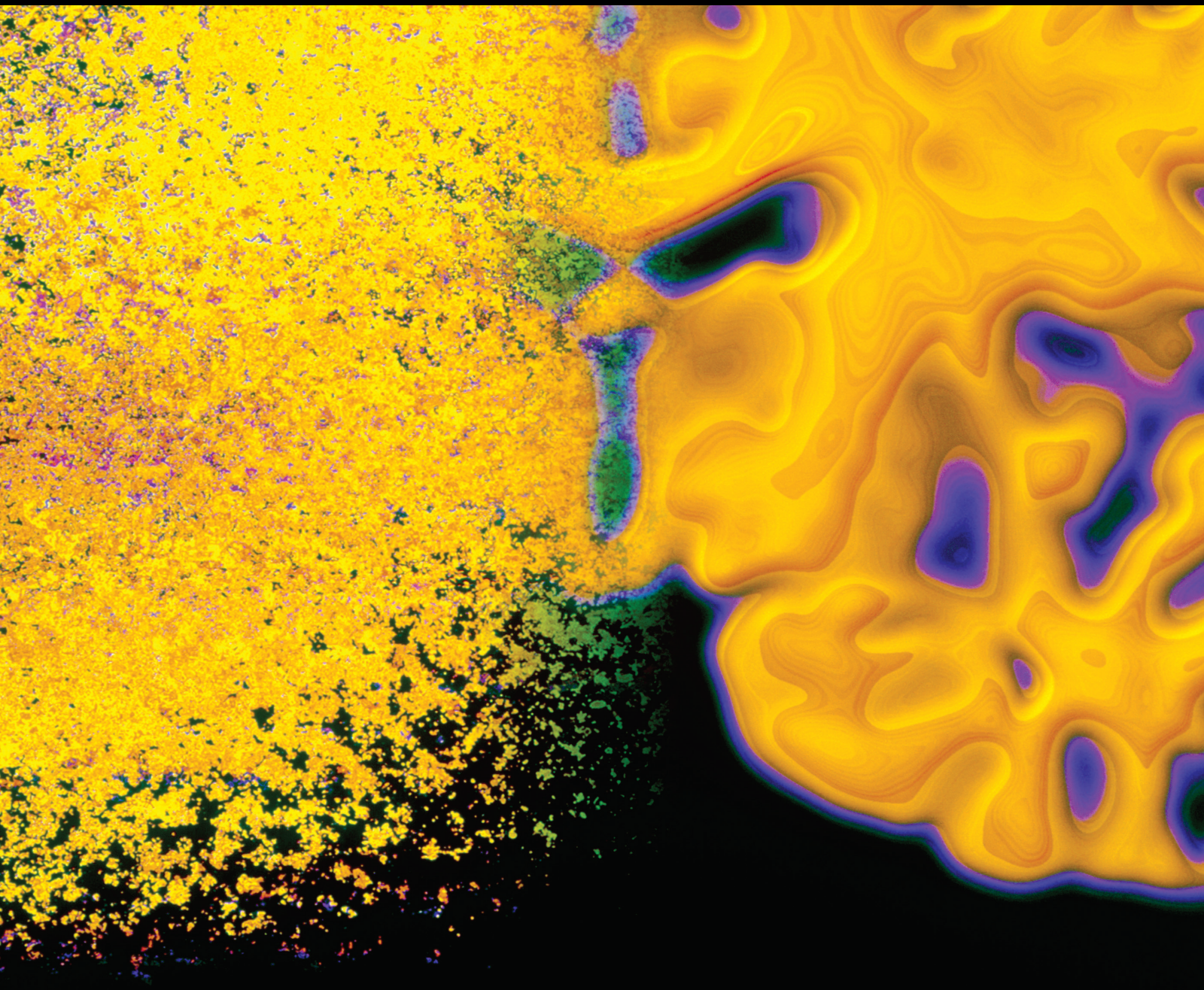

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Contents

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Research Article

The Effects of Poria cocos on Rho Signaling-Induced Regulation of Mobility and F-Actin Aggregation in MK-801-Treated B35 and C6 Cells

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Background and Aim. We recently investigated whether *Poria cocos* water extract modulates ketamine-induced Rho signaling regulation and reverses ketamine-inhibited cell mobility and F-actin reconstruction in B35 and C6 cells. Various studies have mentioned that drugs of abuse induce changes in neuronal plasticity in the brain's reward circuitry. Modulations in neuronal plasticity are closely related to Rho signaling regulation in cells. Rho signaling has also been implicated in the addictive behavior induced by chronic opiate or morphine administration. MK-801 could induce Rho signaling regulation to further modulate cell migration and actin reorganization in neuronal and glial cells. In this study, we investigated the effects of Poria cocos water extract on Rho signal regulation in MK-801-treated B35 and C6 cells. *Methods*. B35 neuronal cells and C6 glial cells were incubated with MK-801 for 7 days followed by MK-801, MK801 in combination with water extracts of P. cocos (PRP for P. cocos cum Radix Pini or WP for White Poria) treatment for an additional 7 days. Analysis of cell mobility, F-actin aggregation, and Rho signaling modulation was performed to clarify the roles of PRP or WP in MK-801-treated B35 and C6 cells. *Results*. MK-801 decreases B35 cell mobility, whereas the inhibited cell migration ability and F-actin aggregation in MK-801-treated B35 or C6 cells could be reversed by PRP or WP. The CDC42 expression in B35 or C6 cells would be reduced by MK-801 and restored by treating with PRP or WP. The RhoA expression was increased by MK-801 in both B35 and C6 cells but was differentially regulated by PRP or WP. In B35 cells, downregulation of PFN1, N-WASP, PAK1, and ARP2/3 induced by MK-801 can be reversely modulated by PRP or WP. PRP or WP reduced the increase in the p-MLC2 expression in B35 cells treated with MK-801. The reduction in ROCK1, PFN1, p-MLC2, and ARP2/3 expression in C6 cells induced by MK-801 was restored by PRP or WP. Reduced N-WASP and PAK1 expression was differentially regulated by PRP or WP in MK-801-treated C6 cells.

1. Introduction

Cytoskeletal reorganization can lead to changes in cell function [1], including elongation of neuronal spines of synapse, cell mobility [2–4], and plasticity of neuron [5, 6]. These cell functions play critical roles in developing neurons and involve the identical regulatory signaling pathway named the Rho signaling pathway. The Rho signaling pathway is triggered by the activation of Rho family proteins and regulates biological functions, such as gene transcription regulation, membrane trafficking, growth/shrinkage of microtubules, and actin cytoskeleton reorganization. The most studied proteins in all Rho family protein members are Ras homolog family member A (RhoA), Ras-related C3 botulinum toxin substrate 1 (Rac1), and cell division cycle 42 (Cdc42), which mediate the formation of stress fibers, lamellipodia formation,

membrane ruffling, the formation of filopodia/microspikes, and neuronal development [7]. A previous study suggested that the abnormality of recognitive function in animals might be caused by the impairment of hippocampal neurons [8]. Schubert et al. [9] mentioned that activated RhoA-GTPase induces the regulation of dendritic spine morphology in cultured hippocampal neurons. A reduction in CDC42 expression levels is accompanied by reduced dendritic spine density in the brains of schizophrenic patients. A recent study also proposed that ketamine-induced reductions in Rho signaling might be related to impairment of cognition in schizophrenic patients [10].

Addictive behaviors have been proposed to be closely related to cognitive function [11, 12]. Drug addiction has also been reported to be an affective-cognitive disorder with dopamine transmission abnormalities [13] and N-methyl-d-aspartate receptor (NMDAR) activity [14]. The neuropathology of drug addiction has been suggested to involve cognitive functions, such as memory, learning, attention, and inhibitory control during the development of drug dependence [15]. Previous studies revealed that ketamine would reduce RhoA and ROCK1 expression levels to further cause a reduction of mushroom spine formation and stubby spine number of hippocampal neurons in rats, which might be related to impaired cognitive function in schizophrenic patients [8, 10, 16–18]. It has also been reported that RhoA might interact with the metabotropic glutamate receptor 1, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, and NMDAR to maintain the stabilization of NMDAR and to modulate the reconstruction of spine actin at excitatory postsynapses [9]. In addition, the regulation of dendritic spine morphology in cultured hippocampal neurons can be induced by activating RhoA [9]. These findings suggested that addictive behaviors might be related to cognitive functions that can be modulated by the Rho signaling pathway.

Poria *cocos* is a traditional Chinese medicine containing two major ingredients, triterpenoids and polysaccharides, and some minor chemical substances, including potassium salts, amino acids, choline, histidine, and steroids [19, 20]. *P. cocos* cum Radix Pini (PRP; Sclerotium paradicis, named *Fu Shen*) and White Poria (WP; named *Bai Fu Ling*) are two commonly used herbal medicines of *P. cocos* with pharmacological anti-inflammatory properties and immunomodulatory properties [21–23]. Several reports mentioned that the extract of *P. cocos* regulates the cytoplasmic free calcium concentration in the brain neurons of neonatal rats and dose-dependently modulates glutamate-induced cytosolic free calcium [19, 23]. Some studies have also suggested that cytoplasmic free calcium in cells can regulate the Rho signaling pathway to further modulate cell functions, such as directed movement, mesoderm migration, cytoskeletal reorganization, neuronal cell plasticity, and cancer metastasis [2, 6, 24–26]. The impairment of specific synaptic plasticity in the mesolimbic dopamine system, which is central to reward processing in the brain, was suggested to be related to drug abuse behaviors [27]. Our previous study revealed that the water extract of *Poria cocos* can modulate cytoskeletal reorganization and cell migration by affecting RhoA and CDC42 and the subsequent Rho signaling pathway. The water extract of *Poria cocos* could also reverse ketamine-induced effects on cytoskeleton reorganization and cell migration by regulating the RhoA, CDC42, and Rho signaling pathways [28].

MK-801 is an NMDA antagonist that can desensitize addictive behaviors to ethanol, morphine, and cocaine [29, 30]. MK-801 can also induce negative symptoms of schizophrenia, such as cognitive disruption, reduction of long-term potentiation, and learning defects [31–33]. Various previous studies have mentioned that glial cells can maintain neuronal cell function by providing cell shape maintenances, nutrients, growth factors, and recycling of neurotransmitters. The differential effects of *Poria cocos* on these two different types of cells should also be clarified to further understand the drug effects exhibited by *P. cocos* on different cells in the brain. In this study, we aimed to investigate the regulatory effects of *P. cocos* (PRP and WP) on Rho signaling pathway regulation in MK-801-treated B35 neuronal cells and C6 glial cells. We revealed that Rho family proteins (RhoA and CDC42) and Rho signaling-related proteins in B35 and C6 cells could be affected by treating with PRP or WP. We also found either of PRP or WP could recover the inhibitory effects on cell mobility and actin aggregation induced by MK-801 in B35 or C6 cells. Our data proposed that PRP or WP-induced regulation of cell mobility and F-actin reorganization in B35 and C6 cells treated with MK-801 might be caused by reversely modulating RhoA, CDC42, and further Rho signaling pathway regulation in cells.

2. Materials and Methods

2.1. PRP and WP Water Extract Preparation. PRP and WP herbal powder extracts were purchased from Sun Ten Pharmaceutical Company (New Taipei City, Taiwan). For the preparation of the PRP and WP solutions, the PRP or WP herbal powders were weighed and extracted using sterilized ddH2O for 6 h at room temperature. The stock concentration of each of the PRP and WP was adjusted to10 mg/ml. After centrifugation, collect the corresponding supernatant from the PRP and WP solution and store −20°C for future use.

2.2. B35 and C6 Cell Culture and Drug Treatments. MK-801 was purchased from Sigma–Aldrich (St. Louis, USA). B35 and C6 cells were obtained from the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (FRDI), Taiwan. For B35 cells, cells were cultured in MEM (Invitrogen, Life Technologies) containing 10% fetal bovine serum (Invitrogen, Life Technologies), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. High-glucose Dulbecco's Modified Eagle's Medium (Invitrogen, Life Technologies Incorporation, Eugene, OR, USA) supplemented with 2 mM L-glutamine, 2% fetal bovine serum (Invitrogen Life Technologies) and 10% heat-inactivated horse serum (Invitrogen Life Technologies) were used to maintain C6 cells throughout the experiments. To examine the effects of MK-801, PRP, and WP on B35 and C6 cells, cells were treated with MK-

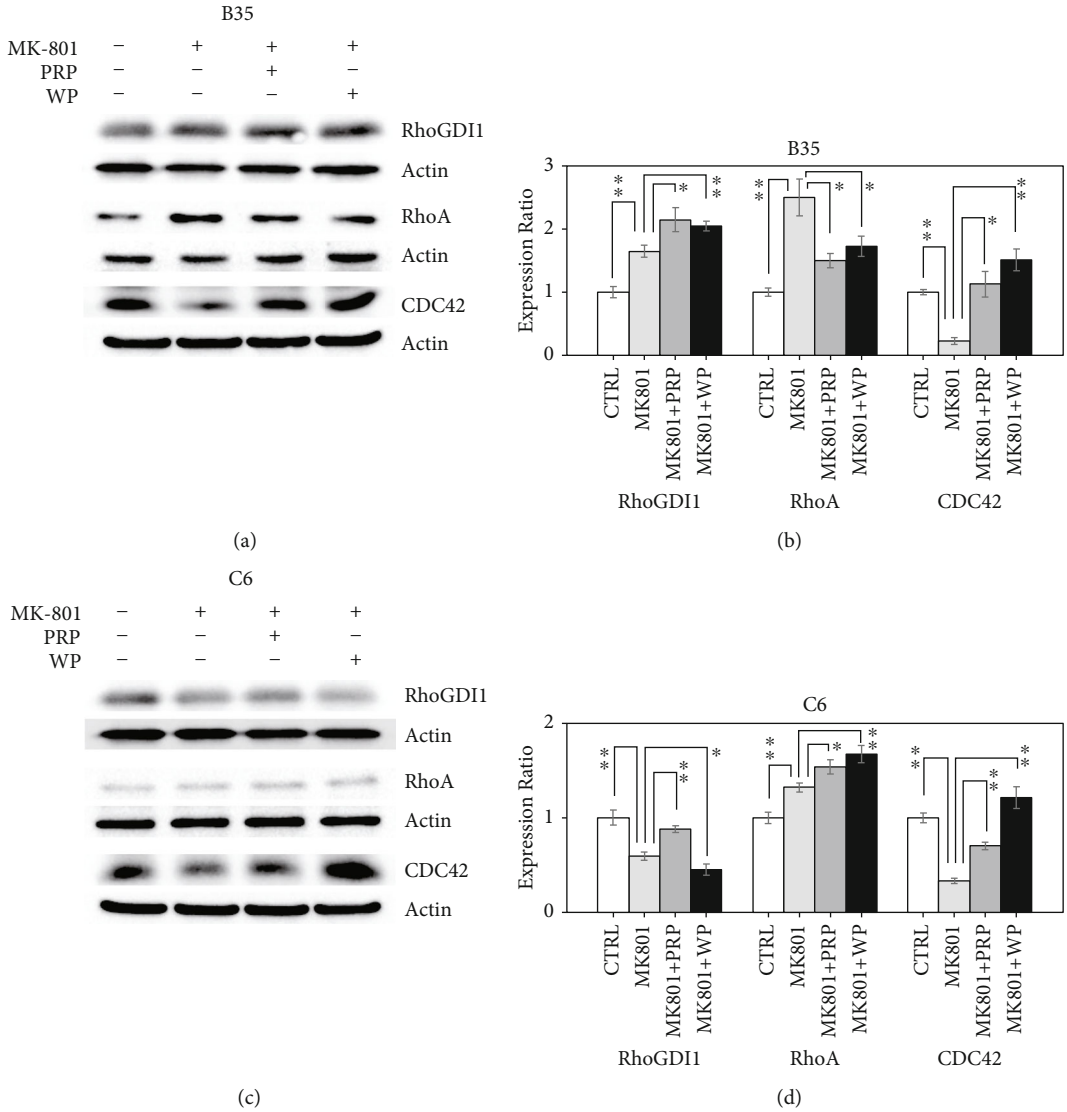

Figure 1: PRP and WP differentially regulate MK-801-induced RhoGDI1, RhoA, and CDC42 regulation. Western blotting revealed the expression changes of RhoGDI1, RhoA, and CDC42 induced by PRP/WP in (a) B35 and (c) C6 cells treated with MK-801. The protein expression was quantified by using ImageJ software. Beta-actin was used as a normalization control to calculate the relative expression of the examined targets. The bar chart was constructed according to the data of three independent western blot experiments that analyzed three different batches of protein extracts of drug-treated (b) B35 and (d) C6 cells. The data was analyzed by using Student's t-test ($^{*}p$ value<0.05; $^{**}p$ value<0.01) analysis.

801 daily for 7 days followed by the addition of MK-801 alone or in combination with either PRP or WP for an additional 7 days. The final drug concentration used was 25 μM for MK-801, 10 μg/ml for PRP, and 10 μg/ml for WP. B35 or C6 cells were then collected for extracting total protein, followed by subsequent expression level analysis of RhoGDI1, Rho family proteins (RhoA and CDC42), and Rho signaling pathway-related proteins (ROCK1, PFN1, p-MLC2, N-WASP, ARP2/3, and PAK1).

2.3. Western Blot Analysis. Total cell lysates were prepared by lysing B35 or C6 cells in mammalian protein extraction buffer (GE Healthcare Bio-Science, Uppsala, Sweden) supplemented with Protease Inhibitor Mix (GE Healthcare Bio-Science). To examine the protein expression levels, 5–80 μg of total protein extract of B35 or C6 was analyzed by separating in 10-15% sodium dodecyl sulfate polyacrylamide gels accordingly. The polyvinylidene difluoride membranes were used for transferring separated proteins. The membrane was then blocked with Membrane Blocking Solution (Life Technology, Frederick, MD, USA) for 1 h. The blocked membranes were then incubated with specific primary antibodies at 4°C for 12 h, followed by incubation with the respective horseradish peroxidase-conjugated secondary antibodies at room temperature for 4 h. Amersham ECL kit (Amersham, Bucks, UK) was used to develop and to reveal the signals of protein bands.

2.4. Mobility Analysis of B35 and C6 Cells. Before performing the cell mobility assay, B35 or C6 cells were cultured in

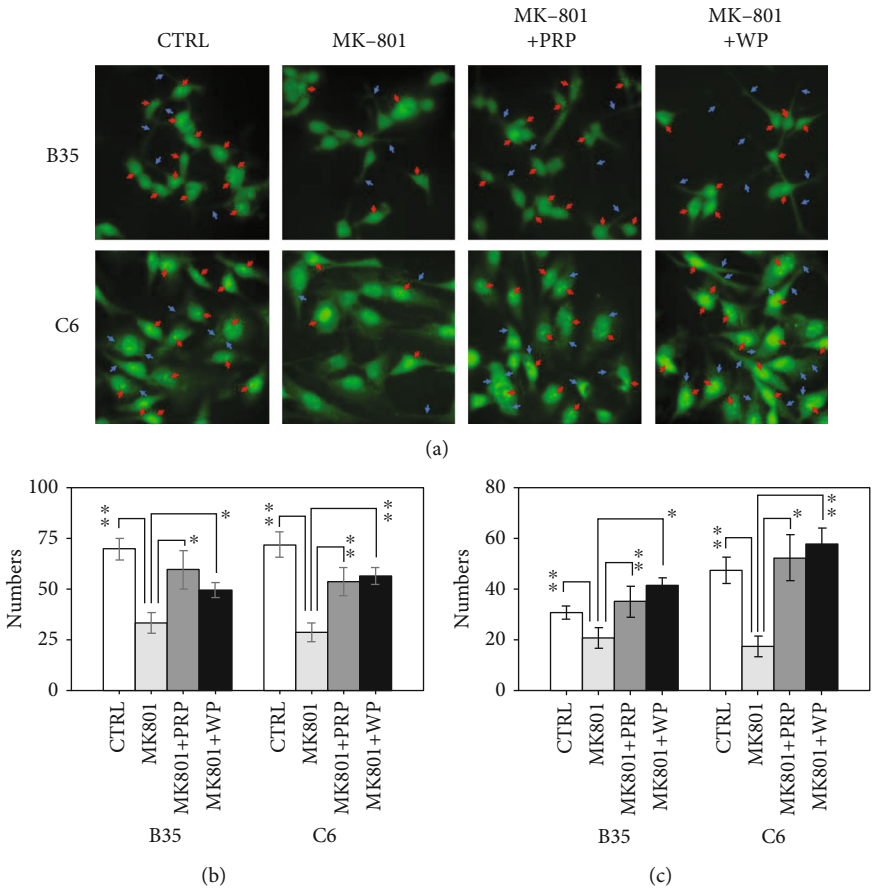

Figure 2: Effects of PRP and WP on actin filament reorganization in B35 and C6 cells treated with MK-801. MK-801-treated B35 and C6 cells on coverslips were incubated with PRP or WP accordingly. ActinGreen™ 488 RreadyProbes™ reagent was used for F-actin in staining in B35 and C6 cells. (a) Red arrows show actin nucleation and blue arrows show F-actin condensation. The numbers of (b) actin nucleation and (c) F-actin condensation were obtained by counting the condensed actin spot in nuclei or condensed actin filament in extended-cytoplasm in 100 cells under different fields of view. The bar charts were made from three independent batches of experiments and analyzed by using Student's t-test ($^{*}p$ value <0.05; $^{**}p$ value <0.01) analysis.

medium with MK-801 for 7 days, followed by treating with MK-801 combined with PRP or WP for another 5 days. Drug-treated B35 cells (10^4 cells/well) or drug-treated C6 (5×10^3 cells/well) were added to the upper Transwell (Costar, Coring Incorporation, Kennebunk, ME, USA) insert compartment with an 8 μm pore size polycarbonate membrane. Cells in Transwell insert were then incubated in a 24-well tissue culture plate with medium containing MK-801 and either PRP or WP for another 2 days. Cell migration assays were performed by fixing B35 or C6 cells with methanol and followed by staining the cells with a propidium iodide solution (Sigma) (50 μg/ml) and staying at room temperature for 45 min. The migrated cells on the other side of the membrane were counted using a fluorescent microscope at ×40 magnification. The mobility assay for all experiments in this study was performed independently in triplicate.

2.5. Analysis of Actin Condensation in B35 and C6 Cells. B35 or C6 cells were cultured with MK-801 for 7 days and then incubated with MK-801 in combination with or without PRP/WP for another 5 days. 5×10^3 drug-treated B35 or C6 cells were then seeded in 6-well plate coverslips coated with poly-L-lysine. The cells were further cultured for another 2 days in medium with corresponding MK-801, PRP, or WP. The coverslips with drug-treated B35 or C6 cells were moved to a new 6-well plate and fixed by incubating the coverslips in methanol for 2 h at -20°C. After washing with PBS, the cells on coverslips were stained by immersing in ActinGreen™ 488 RreadyProbes™ reagent (Invitrogen, Life Technologies) at room temperature for 90 min according to the manufacturer's suggestion. The traced dye was washed out from the cells by using PBS, and the coverslips were moved to glass slides and mounted with SlowFade™ Diamond Antifade Mountant (Invitrogen, Life Technologies). The images were then captured on a fluorescent microscope at ×40 magnification.

2.6. Quantification of Protein Expression Level and Statistical Analysis. The expression level of the examined proteins in all western blot experiments was obtained by detecting the density of developed bands using ImageJ software (https://imagej.nih.gov/ij/). Differences in normalized protein expression levels and cell migration assays between MK-

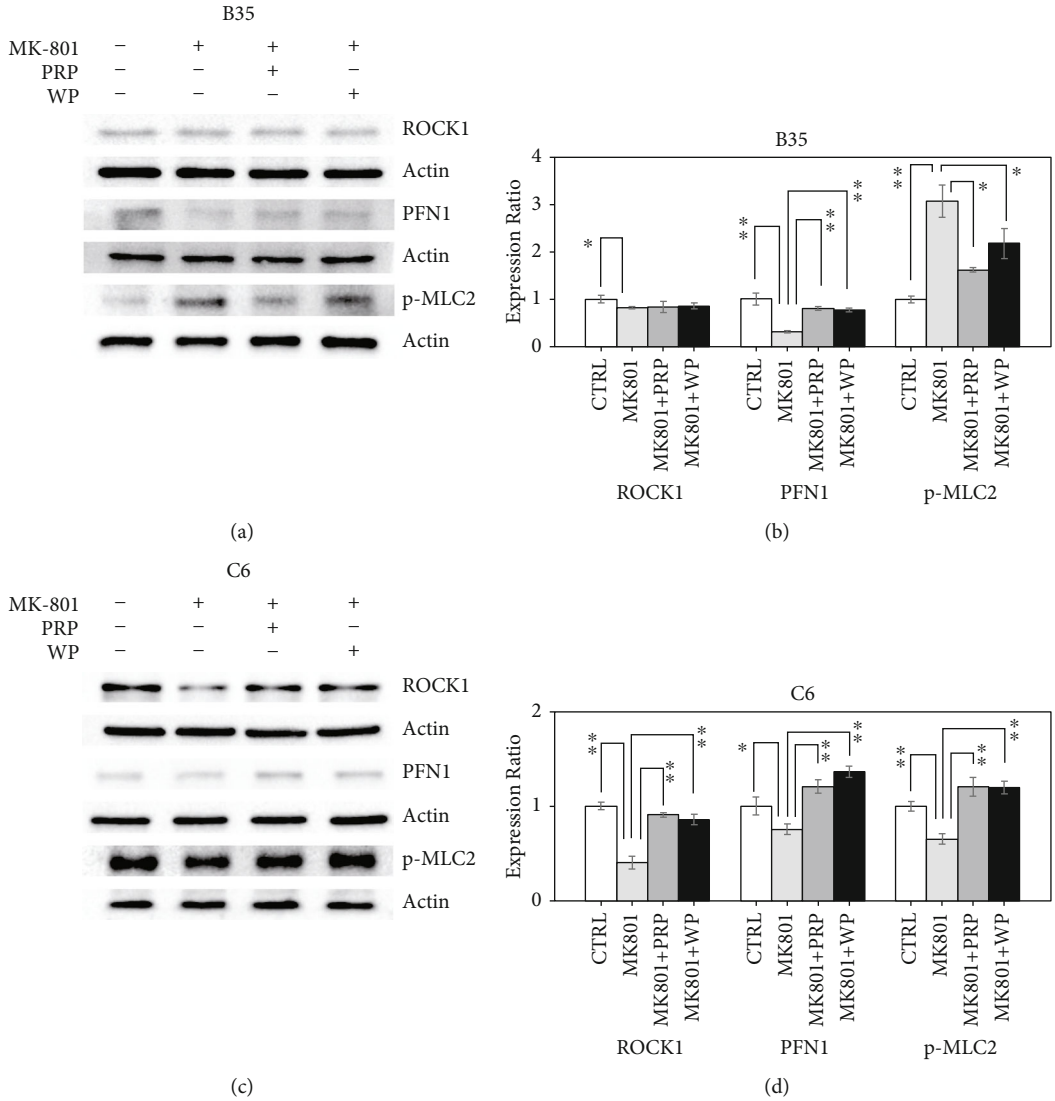

Figure 3: PRP and WP regulate MK-801-induced ROCK1, PFN1, and p-MLC2 regulation. Western blotting revealed the expression changes of ROCK1, PFN1, and p-MLC2 induced by PRP/WP in (a) B35 and (c) C6 cells treated with MK-801. The protein expression was quantified by using ImageJ software. Beta-actin was used as a normalization control to calculate relative expression of examined target. The bar chart was constructed according to the data of three independent western blot experiments that analyzed three different batches of protein extracts of drug-treated (b) B35 and (d) C6 cells. The data was analyzed by using Student's t-test ($^{*}p$ value <0.05; $^{**}p$ value <0.01) analysis.

801 and control, differential drug-treated B35 and C6 cells were analyzed by using Student's t-test. A p value less than 0.01 (**) or 0.05 (*) was used to represent significant differences between compared groups.

3. Results

3.1. PRP and WP Modulate MK-801-Induced RhoGDI1, RhoA, and CDC42 Regulation. Our previous findings revealed that PRP and WP could modulate the expression levels of RhoGDI1 and RhoA and CDC42 but not Rac1 proteins [28]. In the present study, we examined the effects of PRP and WP on modulating the RhoGDI1, RhoA, and CDC42 protein expression in MK-801-treated B35 and C6 cells. We found that MK-801 increased the RhoGDI1 expression (p value <0.01) and was further increased by PRP (p value<0.05) and WP (p value <0.01) in B35 cells (Figures 1(a) and 1(b)). The increased RhoA expression induced by MK-801 (p value <0.01) in B35 cells was restored by PRP (p value <0.05) and WP (p value <0.05) (Figures 1(a) and 1(b)). In contrast, we found that the MK-801-induced reduction in the RhoGDI1 expression (p value <0.01) was recovered by PRP (p value <0.01) but was further reduced by WP (p value <0.05) in C6 cells (Figures 1(c) and 1(d)). The RhoA expression induced by MK-801 treatment in C6 cells (p value <0.01) was further increased by PRP (p value <0.05) and WP (p value <0.01) (Figures 1(c) and 1(d)). The reduction in the CDC42 expression in MK-801-treated B35 (p value <0.01) and C6 (p value <0.01) cells was recovered by either PRP (p value <0.05 for B35 and p value <0.01

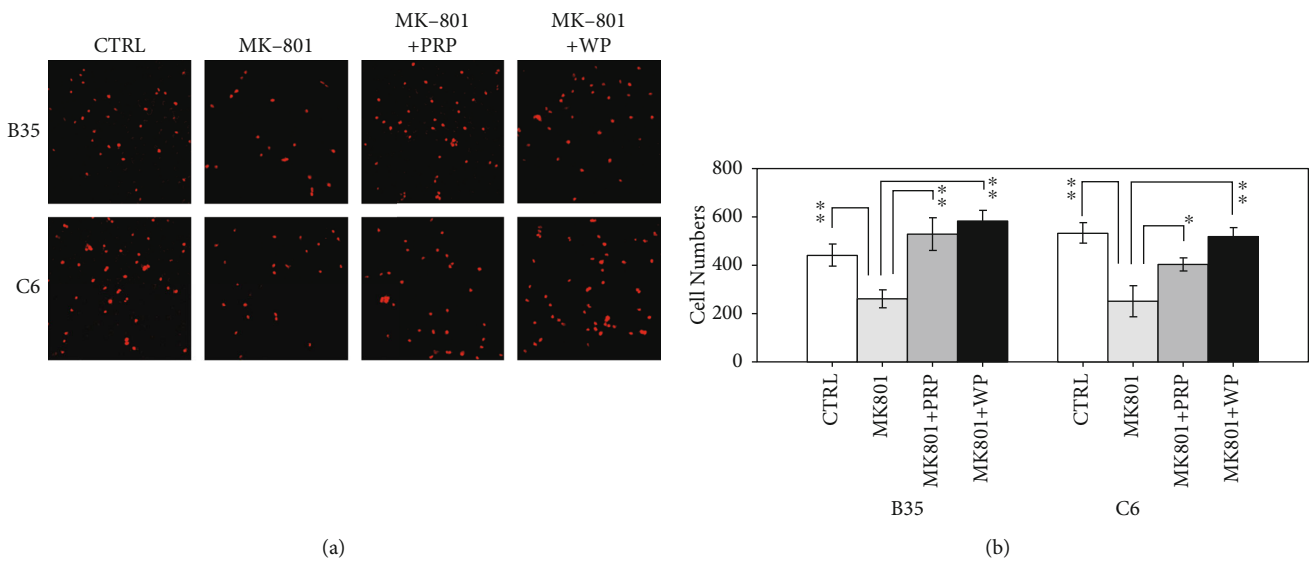

Figure 4: Effects of MK-801, PRP, and WP on B35 and C6 cell migration. (a) Cell mobility of B35 and C6 cells was analyzed. The migrated cells were stained with propidium iodide, and then the number of cells on the membrane was counted. (b) The result in the bar chart was made from cell counts of three different drug-treated cell batches and analyzed using Student's t-test ($^{*}p$ value <0.05; $^{**}p$ value <0.01).

for C6) or WP (p value <0.01 for both cells) treatment (Figure 1).

3.2. Effects of PRP and WP on Modulating F-Actin Reorganization in MK-801-Treated B35 and C6 Cells. Actin condensation and cytoskeletal reorganization play important roles in various cell functions mediated by Rho signaling, including actin nucleation/polymerization, regulation of cell shape, microtubule formation, and cell polarity regulation. We observed that MK-801 could reduce actin nucleation in both B35 and C6 cells after staining cells with phalloidin (Figure 2). We also observed a reduction in actin filament formation in B35 and C6 cells upon treatment with MK-801 (Figure 2). Both PRP and WP reversed the inhibitory effects of MK-801 on actin nucleation and F-actin construction in B35 and C6 cells.

3.3. PRP and WP Induced RhoA-Related Rho Signaling Regulation in MK-801-Treated B35 and C6 Cells. In RhoA-regulated Rho signaling pathway, ROCK1, profilin 1 (PFN1), and phosphorylated myosin light chain 2 (p-MLC2) are proteins that can modulate F-actin assembly and condensation. The ROCK1 expression was decreased by MK-801 (p value<0.05) in B35 cells (Figures 3(a) and 3(b)), whereas PRP and WP did not affect the reduced ROCK1 level caused by MK801. The reduction in the ROCK1 expression caused by MK-801 (p value<0.01) in C6 cells could be increased by either PRP (p value <0.01) or WP (p value <0.01) (Figures 3(c) and 3(d)). The reduction in the PNF1 expression induced by MK-801 in B35 (p value <0.01) (Figures 3(a) and 3(b)) and C6 (p value <0.05) (Figures 3(c) and 3(d)) cells could be recovered by PRP (p value <0.01 for both B35 and C6 cells) and WP (p value <0.01 for both B35 and C6 cells). The increased expression of p-MLC2 in MK-801-treated B35 cells (p value <0.01) was reduced by PRP (p value <0.05) and WP (p value <0.05) (Figures 3(a) and 3(b)). In contrast, we observed that MK-801 reduced the p-MLC2 expression (p value <0.01) in C6 cells, which could be recovered by PRP (p value <0.01) and WP (p value <0.01) (Figures 3(c) and 3(d)).

3.4. PRP and WP Restored MK-801-Mediated Inhibition of B35 and C6 Cell Migration. To examine the effect of PRP and WP on MK-801-induced inhibition of B35 and C6 cell migration, B35 or C6 cells were incubated with MK-801 for 7 days followed by PRP or WP treatment for another 6 days. Then, a cell migration assay was performed for an additional 24 h. As shown in Figure 4, B35 and C6 cell migration was inhibited by MK-801 (p value <0.01 for both B35 and C6 cells). However, MK-801-induced inhibition of B35 (p value <0.01 for both PRP and WP) and C6 cell migration (p value <0.01 for both PRP and WP) could be restored by PRP and WP.

3.5. PRP and WP Modulated CDC42-Related Rho Signaling Regulation in MK-801-Treated B35 and C6 Cells. Activation of CDC42 can modulate cell migration ability by regulating neuronal Wiskott–Aldrich syndrome protein (N-WASP), p21 (RAC1)-activated kinase 1 (PAK1), and RhoA protein–modulated actin-related protein 2/3 (ARP2/3) to further regulate various cell functions, including actin polymerization, filopodia, and cell migration. We observed that the reduction in the N-WASP expression in MK-801-treated B35 (Figures 5(a) and 5(b)) and C6 cells (Figures 5(c) and 5(d)) (p value <0.01 for both B35 and C6 cells) was restored by PRP (p value<0.05 for B35 cells and p value <0.01 for C6 cells) but was further reduced by WP (p value <0.01 for both B35 and C6 cells). PRP (p value <0.01) but not WP enhanced the reduction in the PAK1 expression in B35 cells treated with MK-801 (p value

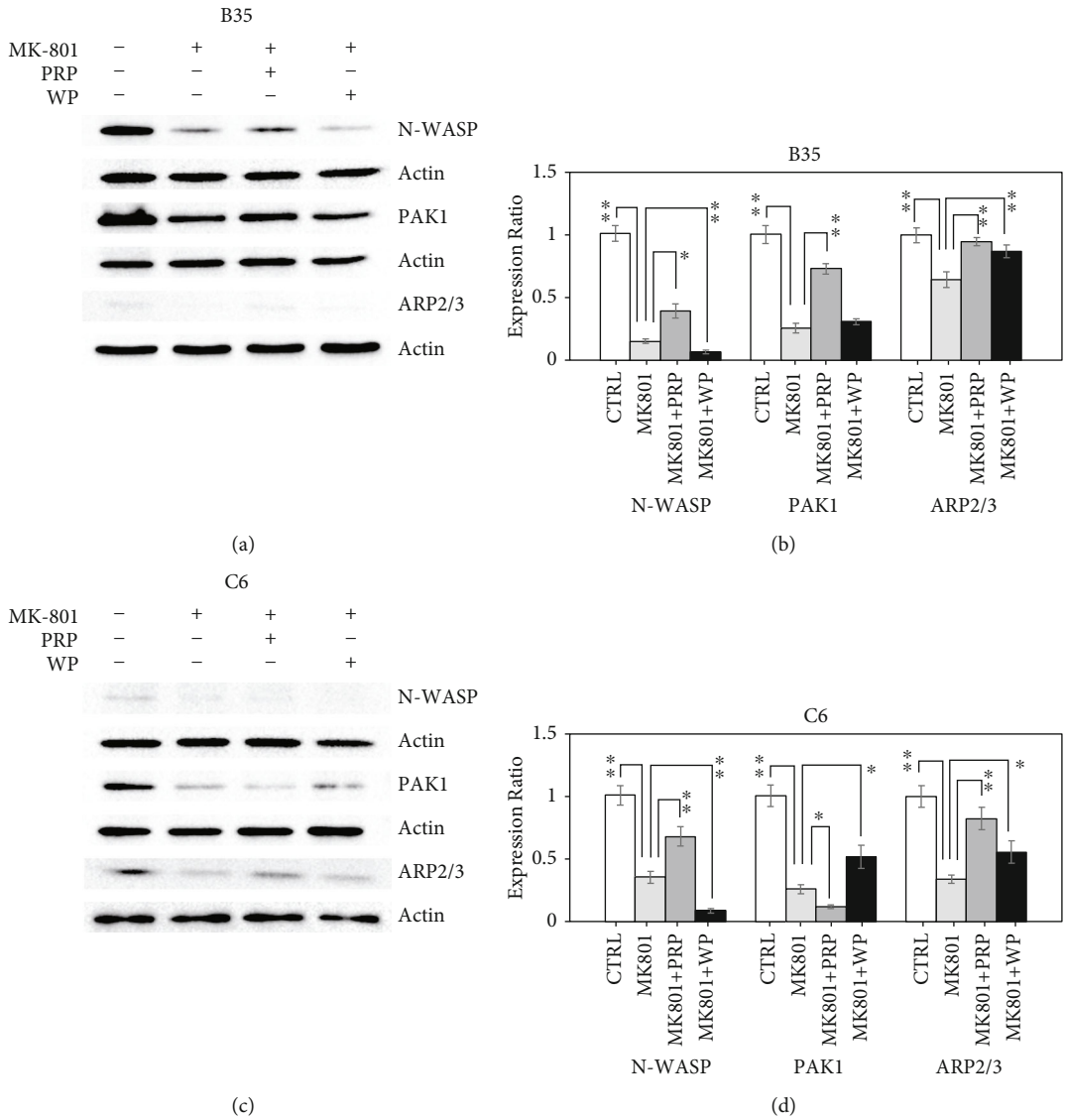

Figure 5: PRP and WP regulate MK-801-induced N-WASP, PAK1, and ARP2/3 regulation. Western blotting revealed the expression changes of the indicated proteins induced by PRP/WP in (a) B35 and (c) C6 cells treated with MK-801. The protein expression was quantified by using ImageJ software. Beta-actin was used as a normalization control to calculate relative expression of examined target. The bar chart was constructed according to the data of three independent western blot experiments that analyzed three different batches of protein extracts of drug-treated (b) B35 and (d) C6 cells. The data was analyzed by using Student's t-test ($^{*}p$ value <0.05; $^{**}p$ value <0.01) analysis.

<0.01) (Figures 5(a) and 5(b)). In MK-801-treated C6 cells, the downregulation of PAK1 (p value <0.05) was further reduced by PRP (p value <0.05) but will be enhanced by WP (p value <0.05) (Figures 5(c) and 5(d)). MK8-01 reduced the ARP2/3 expression in B35 (p value <0.01) (Figures 5(a) and 5(b)) and C6 cells (p value <0.01) (Figures 5(c) and 5(d)), whereas both PRP (p value<0.01 for both B35 and C6 cells) and WP (p value <0.01 for B35 cells and p value <0.05 for C6 cells) restored ARP2/3 expression levels.

4. Discussion

The Rho signaling pathway plays important roles in modulating actin filament construction to regulate various cell functions, such as cell shape changes, cell migration, neuronal cell plasticity, cytoskeleton reorganization, and microtubule formation. The relationship between Rho signaling regulation and neuronal cell plasticity has been mentioned and well studied in various studies. Many studies have also revealed that neuronal cell plasticity is related to the generation of addictive behaviors [27, 34]. MK-801 was found to impair cognitive function, learning ability, and memory and was also used to ease addictive behaviors [29–33, 35]. The present study revealed that MK-801 could enhance the RhoA expression and reduce the CDC42 expression in both B35 and C6 cells. We also found that PRP and WP could reverse the effects of the MK-801 on CDC42 expression in both cell types. This finding suggested that PRP and WP

might regulate CDC42 but not RhoA through a similar mechanism in MK-801-treated B35 and C6 cells.

Although MK-801 was found to ease the addictive behaviors induced by various drugs, MK-801 was also found to impair recognition function by regulating neuronal plasticity and related immediate early gene expression by inhibiting NMDA receptors on pyramidal neurons and axonal boutons in hippocampal interneurons of rats [36–38]. Recently, MK-801 was revealed to decrease AMPA receptors and further metaplasity (plasticity of synaptic plasticity) of neurons [39, 40] related to stress or drugs of abuse. In this study, PRP and WP were found to reverse regulation of RhoA and CDC42 expression level in MK-801-treated B35 neuronal cells. Furthermore, PRP and WP were also found to reverse MK-801-induced PFN1, pMLC2, and ARP2/3 expression in B35 cells. Regulation of N-WASP and PAK1 in MK-801-treated B35 cells could be restored by PRP but not WP. The differential regulation of N-WASP and PAK1 may be caused by the different ingredients of PRP and WP. The regulatory effects of the ingredients PRP and WP on Rho signaling proteins should be further studied.

Activation of ROCK1 protein by activated RhoA might further induce the phosphorylation of p-MLC2. A recent study observed that directly delivering Fasudil, a ROCK protein inhibitor, to the prefrontal cortex of mice might enhance goal-directed behavior and block the habitual response to cocaine [41]. Our study has shown that the MK-801-induced ROCK1 expression was not affected by PRP or WP in B35 cells. Interestingly, we also observed the decreased PFN1 and increased p-MLC2 expression in MK-801-treated B35 cells, which could be reversed by PRP and WP. The regulatory trends were the same as those of RhoA expression regulation. This finding suggested that the ROCK1 expression might be regulated by factors other than RhoA activation.

MK-801 is a NMDAR antagonist that can bind NMDAR and to further induce changes of calcium level in cytoplasm of cells. Although both B35 neuronal cell and C6 glial cell have NMDARs for binding of MK-801, the proportion of NMDARs on the membrane and the modulation of signaling downstream the NMDARs might be varied between different cell types to induce differential RhoGDI1 regulation. These might be the reasons that RhoGDI1 expressions were differentially regulated in B35 cell and C6 cell. In addition, the different effects of PRP and WP on the regulation of the RhoA expression in B35 and C6 cells may be caused by the different susceptibility of the cells to PRP and WP. Furthermore, the ingredients of PRP and WP are similar but still slightly different. This might be the factor that PRP and WP induce differential regulation of RhoGDI1, N-WASP, and PAK1 expression in C6 cells. To determine whether the differences of ingredients between PRP and WP might be the reason to induce differential regulation of RhoGDI1, N-WASP, and PAK1 in B35 and/or in C6 cells should be further studied. Additionally, RhoA, CDC42, and Rac1 separately and dynamically modulate actin filament formation, actin contraction, and lamellipodial protrusions in cell. LIMK was found that can be coregulated by RhoA- and CDC42-related Rho signaling. The crosstalk between RhoA- or CDC42-related signaling via LIMK might be the cause for the inconsistent regulation between ROCK1, PFN1/p-MLC2, N-WASP, and PAK1.

5. Conclusion

The relationships between addictive behaviors and Rho signaling (except RhoA, CDC42, and ROCK1) remain unclear. We conclude that PRP and WP could regulate the RhoA and CDC42 expression to modulate Rho signaling pathway and follow cell migration, actin nucleation, and F-actin remodeling in MK-801-treated B35 and C6 cells. Further studies should be also performed to better clarify the roles of PRP/WP playing on Rho signaling, neuronal plasticity, and addictive behaviors in suitable animal models.

Data Availability

All data used to support the findings of this study are available from the corresponding author upon reasonable request.

Disclosure

All sponsors or funders played no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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References

[1] D. A. Fletcher and R. D. Mullins, "Cell mechanics and the cytoskeleton," *Nature*, vol. 463, no. 7280, pp. 485–492, 2010.

[2] F. C. Tsai, G. H. Kuo, S. W. Chang, and P. J. Tsai, "Ca2+ signaling in cytoskeletal reorganization, cell migration, and cancer metastasis," *BioMed Research International*, vol. 2015, Article ID 409245, 13 pages, 2015.

[3] D. D. Tang and B. D. Gerlach, "The roles and regulation of the actin cytoskeleton, intermediate filaments and microtubules in smooth muscle cell migration," *Respiratory Research*, vol. 18, no. 1, p. 54, 2017.

[4] D. Yamazaki, S. Kurisu, and T. Takenawa, "Regulation of cancer cell motility through actin reorganization," *Cancer Science*, vol. 96, no. 7, pp. 379–386, 2005.

[5] Y. Nakahata and R. Yasuda, "Plasticity of spine structure: local signaling, translation and cytoskeletal reorganization," *Frontiers in Synaptic Neuroscience*, vol. 10, p. 29, 2018.

[6] T. Kuriu, A. Inoue, H. Bito, K. Sobue, and S. Okabe, "Differential control of postsynaptic density scaffolds via actin-dependent and -independent mechanisms," *Journal of Neuroscience*, vol. 26, no. 29, pp. 7693–7706, 2006.

[7] A. Hall, "Rho GTPases and the actin cytoskeleton," *Science*, vol. 279, no. 5350, pp. 509–514, 1998.

[8] A. S. Ivy, C. S. Rex, Y. Chen et al., "Hippocampal dysfunction and cognitive impairments provoked by chronic early-life stress involve excessive activation of CRH receptors," *Journal of Neuroscience*, vol. 30, no. 39, pp. 13005–13015, 2010.

[9] V. Schubert, J. S. Da Silva, and C. G. Dotti, "Localized recruitment and activation of RhoA underlies dendritic spine morphology in a glutamate receptor-dependent manner," *The Journal of Cell Biology*, vol. 172, no. 3, pp. 453–467, 2006.

[10] S. Jiang, Z. Hao, X. Li et al., "Ketamine destabilizes growth of dendritic spines in developing hippocampal neurons in vitro via a Rhodependent mechanism," *Molecular Medicine Reports*, vol. 18, no. 6, pp. 5037–5043, 2018.

[11] T. J. Gould, "Addiction and cognition," *Addiction Science & Clinical Practice*, vol. 5, no. 2, pp. 4–14, 2010.

[12] M. S. D'Souza, "Brain and cognition for addiction medicine: from prevention to recovery neural substrates for treatment of psychostimulant-induced Cognitive deficits," *Frontiers in Psychiatry*, vol. 10, p. 509, 2019.

[13] L. Fattore and M. Diana, "Drug addiction: an affective-cognitive disorder in need of a cure," *Neuroscience & Biobehavioral Reviews*, vol. 65, pp. 341–361, 2016.

[14] F. W. Hopf, "Do specific NMDA receptor subunits act as gateways for addictive behaviors?," *Genes, Brain and Behavior*, vol. 16, no. 1, pp. 118–138, 2017.

[15] K. D. Ersche and B. J. Sahakian, "The neuropsychology of amphetamine and opiate dependence: implications for treatment," *Neuropsychology Review*, vol. 17, no. 3, pp. 317–336, 2007.

[16] H. Buchtova, I. Fajnerova, A. Stuchlik, and S. Kubik, "Acute systemic MK-801 induced functional uncoupling between hippocampal areas CA3 and CA1 with distant effect in the retrosplenial cortex," *Hippocampus*, vol. 27, no. 2, pp. 134–144, 2017.

[17] D. Han, L. Xu, H. Xiao, G. C. Prado Schmidt, and S. Shi, "Dizocilpine reduces head diameter of dendritic spines in the hippocampus of adolescent rats," *Psychiatry Research*, vol. 210, no. 1, pp. 351–356, 2013.

[18] B. Juliandi, K. Tanemura, K. Igarashi et al., "Reduced adult hippocampal neurogenesis and cognitive impairments following prenatal treatment of the antiepileptic drug valproic acid," *Stem Cell Reports*, vol. 5, no. 6, pp. 996–1009, 2015.

[19] J. L. Rios, "Chemical constituents and pharmacological properties of Poria cocos," *Planta Medica*, vol. 77, no. 7, pp. 681–691, 2011.

[20] Y. L. Feng, Y. Y. Zhao, F. Ding et al., "Chemical constituents of surface layer of Poria cocos and their pharmacological properties (I)," *Zhongguo Zhong Yao Za Zhi= Zhongguo Zhongyao Zazhi= China journal of Chinese Materia Medica*, vol. 38, no. 7, pp. 1098–1102, 2013.

[21] M. J. Cuellar, R. M. Giner, M. C. Recio, M. J. Just, S. Manez, and J. L. Rios, "Effect of the basidiomycete Poria cocos on experimental dermatitis and other inflammatory conditions," *Chemical and Pharmaceutical Bulletin*, vol. 45, no. 3, pp. 492–494, 1997.

[22] K. Spelman, J. Burns, D. Nichols, N. Winters, S. Ottersberg, and M. Tenborg, "Modulation of cytokine expression by traditional medicines: a review of herbal immunomodulators," *Alternative Medicine Review*, vol. 11, no. 2, pp. 128–150, 2006.

[23] S. J. Yu and J. Tseng, "Fu-Ling, a Chinese herbal drug, modulates cytokine secretion by human peripheral blood monocytes," *International Journal of Immunopharmacology*, vol. 18, no. 1, pp. 37–44, 1996.

[24] K. Hayashi, T. S. Yamamoto, and N. Ueno, "Intracellular calcium signal at the leading edge regulates mesodermal sheet migration during Xenopus gastrulation," *Scientific Reports*, vol. 8, no. 1, p. 2433, 2018.

[25] C. Wei, X. Wang, M. Zheng, and H. Cheng, "Calcium gradients underlying cell migration," *Current Opinion in Cell Biology*, vol. 24, no. 2, pp. 254–261, 2012.

[26] S. Sakurada, N. Takuwa, N. Sugimoto et al., "Ca2+-dependent activation of Rho and Rho kinase in membrane depolarization-induced and receptor stimulation-induced vascular smooth muscle contraction," *Circulation Research*, vol. 93, no. 6, pp. 548–556, 2003.

[27] J. A. Kauer and R. C. Malenka, "Synaptic plasticity and addiction," *Nature Reviews Neuroscience*, vol. 8, no. 11, pp. 844–858, 2007.

[28] C. Y. Lee, C. T. Lee, I. S. Tzeng, C. Y. Kuo, F. M. Tsai, and M. L. Chen, "Poria cocos regulates cell migration and actin filament aggregation in B35 and C6 cells by modulating the RhoA, CDC42, and Rho signaling pathways," *Evidence-Based Complementary and Alternative Medicine*, vol. 2021, Article ID 6854860, 12 pages, 2021.

[29] R. Camarini, R. Frussa-Filho, M. G. Monteiro, and H. M. Calil, "MK-801 blocks the development of behavioral sensitization to the ethanol," *Alcoholism: Clinical and Experimental Research*, vol. 24, no. 3, pp. 285–290, 2000.

[30] M. Jeziorski, F. J. White, and M. E. Wolf, "MK-801 prevents the development of behavioral sensitization during repeated morphine administration," *Synapse*, vol. 16, no. 2, pp. 137–147, 1994.

[31] E. J. Coan, W. Saywood, and G. L. Collingridge, "MK-801 blocks NMDA receptor-mediated synaptic transmission and long term potentiation in rat hippocampal slices," *Neuroscience Letters*, vol. 80, no. 1, pp. 111–114, 1987.

[32] T. K. Murray, R. M. Ridley, M. F. Snape, and A. J. Cross, "The effect of dizocilpine (MK-801) on spatial and visual discrimination tasks in the rat," *Behavioural Pharmacology*, vol. 6, pp. 540–549, 1995.

[33] T. K. Murray and R. M. Ridley, "The effect of dizocilpine (MK-801) on conditional discrimination learning in the rat," *Behavioural Pharmacology*, vol. 8, no. 5, pp. 383–388, 1997.

[34] M. Mameli and C. Luscher, "Synaptic plasticity and addiction: learning mechanisms gone awry," *Neuropharmacology*, vol. 61, no. 7, pp. 1052–1059, 2011.

[35] T. E. Brown, B. R. Lee, and B. A. Sorg, "The NMDA antagonist MK-801 disrupts reconsolidation of a cocaine-associated memory for conditioned place preference but not for self-administration in rats," *Learning & Memory*, vol. 15, no. 12, pp. 857–865, 2008.

[36] J. Czerniawski, F. Ree, C. Chia, K. Ramamoorthi, Y. Kumata, and T. A. Otto, "The importance of having Arc: expression of the immediate-early gene Arc is required for hippocampus-dependent fear conditioning and blocked by NMDA receptor antagonism," *Journal of Neuroscience*, vol. 31, no. 31, pp. 11200–11207, 2011.

[37] Š. Kubík, H. Buchtová, K. Valeš, and A. Stuchlík, "MK-801 impairs cognitive coordination on a rotating arena (carousel) and contextual specificity of hippocampal immediate-early

gene expression in a rat model of psychosis," *Frontiers in Behavioral Neuroscience*, vol. 8, p. 75, 2014.

[38] M. Perez-Rando, E. Castillo-Gómez, R. Guirado et al., "NMDA receptors regulate the structural plasticity of spines and axonal boutons in jippocampal interneurons," *Frontiers in Cellular Neuroscience*, vol. 11, p. 166, 2017.

[39] A. Piva, L. Caffino, F. Mottarlini et al., "Metaplastic effects of ketamine and MK-801 on glutamate receptors expression in rRat medial prefrontal cortex and hippocampus," *Molecular Neurobiology*, vol. 58, no. 7, pp. 3443–3456, 2021.

[40] J. Zhao, Y. Peng, Z. Xu et al., "Synaptic metaplasticity through NMDA receptor lateral diffusion," *Journal of Neuroscience*, vol. 28, no. 12, pp. 3060–3070, 2008.

[41] L. M. DePoy, K. S. Zimmermann, P. J. Marvar, and S. L. Gourley, "Induction and blockade of adolescent cocaine-induced habits," *Biological Psychiatry*, vol. 81, no. 7, pp. 595–605, 2017.

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Review Article

Impact of the Aversive Effects of Drugs on Their Use and Abuse

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Drug use and abuse are complex issues in that the basis of each may involve different determinants and consequences, and the transition from one to the other may be equally multifaceted. A recent model of the addiction cycle (as proposed by Koob and his colleagues) illustrates how drug-taking patterns transition from impulsive (acute use) to compulsive (chronic use) as a function of various neuroadaptations leading to the downregulation of DA systems, upregulation of stress systems, and the dysregulation of the prefrontal/orbitofrontal cortex. Although the nature of reinforcement in the initiation and mediation of these effects may differ (positive vs. negative), the role of reinforcement in drug intake (acute and chronic) is well characterized. However, drugs of abuse have other stimulus properties that may be important in their use and abuse. One such property is their aversive effects that limit drug intake instead of initiating and maintaining it. Evidence of such effects comes from both clinical and preclinical populations. In support of this position, the present review describes the aversive effects of drugs (assessed primarily in conditioned taste aversion learning), the fact that they occur concurrently with reward as assessed in combined taste aversion/place preference designs, the role of aversive effects in drug-taking (in balance with their rewarding effects), the dissociation of these affective properties in that they can be affected in different ways by the same manipulations, and the impact of various parametric, experiential, and subject factors on the aversive effects of drugs and the consequent impact of these factors on their use and abuse potential.

1. Drug Use and Abuse

According to recent results from Monitoring the Future (MTF, 2021), a national survey on drug use by 8th, 10th, and 12th graders, 27.3% of students (averaged across grades) reported use of illicit drugs in the past year [1] (for more recent unpublished findings, see https://www.drugabuse.gov/drug-topics/trends-statistics/monitoring-future). In a sample of participants aged 12 years and older in 2020, the National Survey of Drug Use and Health (NSDUH, 2020) found that 13.5% used an illicit drug in the past month [2]. These surveys clearly indicate that a variety of drugs are used, but importantly, they also indicate that a smaller subset of individuals abuse these same drugs. For example, MTF reported patterns and amounts of drug intake generally associated with abuse. Specifically, daily marijuana prevalence in 2020 was at 1.1%, 4.4%, and 6.9%, and binge drinking (defined as at least 5 or more drinks in a row at least once in the past two weeks) was at 5%, 10%, and 17% for 8th, 10th, and 12th graders, respectively [1]. Related findings have been reported by the NSDUH that found that 40.3 million people had a past year substance use disorder [2] as defined by the *Diagnostic and Statistical Manual of Mental Disorders (DSM-5)* (see [2] for the basis of the dramatic difference in the rates of drug abuse when diagnoses are based on the DSM-4 vs. the DSM-5). Further, according to the World Drug Report (2021), the Global Burden of Disease Study (GBD) in 2019 found that substance use disorders accounted for the largest portion of disability-adjusted life years (DALYs), a measure of disease burden taken from the combination of both the number of years of life lost because of premature death and the number of years of life lived with disability [3]. In fact, drug use disorders accounted for 59% of DALYs with approximately 18.1 million years of "healthy" life lost due to disabilities or premature death [3]. Interestingly, among people aged 12 or

older, only 1.4% received any treatment for substance use [2].

2. Allostatic Model of Drug Use and Abuse

Given the multiple causes and consequences of drug use and abuse, understanding this complexity is critical to prevention and treatment strategies [4]. One comprehensive model of these issues has recently been presented by Koob and his colleagues who describe the various stages of drug use and abuse, the factors important in their display, and the neurobiological substrates of each (as well as the role of these substrates in the transition from use to abuse, drug maintenance, and relapse; see [4–7]). Specifically, Koob and his colleagues describe a neuroadaptation model of addiction that consists of three distinct stages: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation, which differ significantly between acute and chronic drug use (see Figure 1).

Acute use represents a pattern of drug intake in the majority of the population using drugs (roughly between 85 and 90%) that is more impulsive and controlled. Application of Koob's model to individuals in this group reveals a specific characterization of the effects of the drug (binge/intoxication), the affective state of the individual following the cessation of the drug effect (withdrawal/negative affect), and the subsequent desire for the drug in its absence (preoccupation/anticipation). As noted in Figure 1, for acute use (the impulsive condition), the drug itself is rewarding, generating an effect preferred by the user (i.e., a rewarding effect). After the drug's effects have subsided, there is no change in the user's affective state, i.e., the user is relatively neutral in the drug's absence. Finally, there is no true craving for the drug in this group, but if such anticipation of the drug does occur, it is one of a desire to repeat its rewarding effects. Importantly, the drug does initiate a compensatory response (allostasis) that generally is opposite in nature to that of its initial effect. If the user takes the drug relatively infrequently, at low doses, and by routes of administration that have a slow onset and offset, this allostatic state subsides and there is no appreciable change in the abovementioned characterization. The drug maintains its reinforcing effects with little change with its absence and no appreciable craving.

However, if the pattern of drug use changes, e.g., increased frequency of use at higher doses and by routes of administration with more rapid onset (and offset), neuroadaptations occur that drive the transition from use to abuse (involving roughly 10-15% of individual drug users). These neuroadaptations move impulsive use to compulsive use where an individual loses control over abstaining from the drug, escalates drug intake, and relapses [5]. For this group of individuals, the drug may still have positively reinforcing effects, although they are likely to be diminished as a result of the drug-induced downregulation of mesolimbic and mesocortical pathways [7] that mediate these effects. This tolerance induces escalation of drug intake. Further, because these systems are involved in regulating natural reward, their diminished state (a compensatory reaction to elevated drug intake) results in a negative affect (anhedonia-dysphoria,

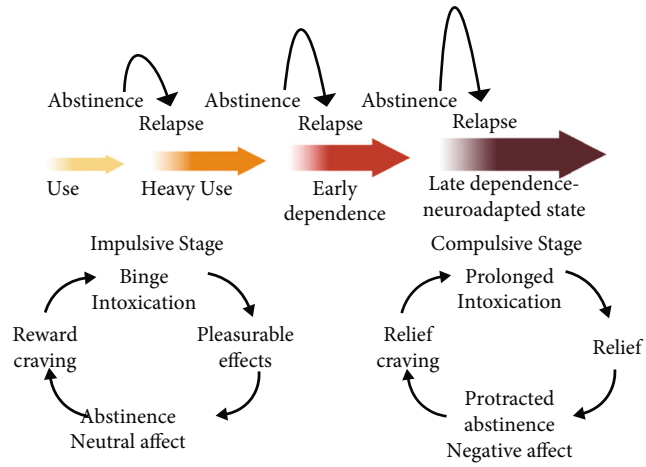

Figure 1: Transition from the impulsive (acute) to the compulsive (abuse) patterns of drug-taking. Adapted from Meyer and Quenzer [8] using BioRender.com.

anxiety, irritability, and sleep disturbances) when the drug is no longer present. This negative affect drives further drug intake by negative reinforcement which is exacerbated by sensitized brain stress systems (primarily in the extended amygdala, i.e., central nucleus of the amygdala, the bed nucleus of the stria terminalis, and a transition zone in the nucleus accumbens) that reflect further compensation to elevated intake. Finally, these individuals now crave the drug when it is absent as the negative affect grows with time since taking the drug. Intake is increased as well by neuroadaptations in yet other systems, e.g., orbitofrontal cortex, which normally mediates salience for traditional reinforcers such as food and sex, is now shifted toward the drug, and the systems involved in executive function (prefrontal cortex; planning, inhibition, memory, and attention) are downregulated. Consequently, these individuals have difficulty targeting relevant reinforcers and inhibiting further drug intake. The cycle continues and spirals out of control with escalated intake, more frequent use, and high rates of relapse (potentiated by the presence of cues and stress that reactivate the mesolimbic and cortical areas via increased input from the frontal cortex).

3. Role of Reward in Drug Use and Abuse

The addiction cycle proposed by Koob and his colleagues [5, 7] illustrates how drug-taking patterns transition from impulsive (acute use) to compulsive (chronic use) as a function of neuroadaptations leading to the downregulation of dopamine pathways and processes, upregulation of stress systems, and the dysregulation of the prefrontal cortex (see above). Important to this analysis is that although the nature of reinforcement initiating and mediating these effects differs (positive vs. negative), the general role of reinforcement in drug intake (both acute and chronic) is well characterized [9–12]. However, drugs have other stimulus properties that may be important as well in drug use and abuse. One such property is a drug's aversive effect that limits drug-taking instead of initiating and maintaining it. The evidence for

such effects comes from both clinical and preclinical research (for an excellent review of how initial responses to a drug impact subsequent use in both clinical and preclinical populations, see [13]).

4. Clinical Evidence of the Aversive Effects of Drugs of Abuse

Clinical anecdotal reports note that drugs have both rewarding and aversive effects (with their use a function of the balance of these two properties; see [14] for a discussion; for factors impacting drug intake, see [9, 13, 15]). For example, smokers often report the first exposure to nicotine as aversive (heart palpitations, feeling faint, dizziness, throat irritation, coughing, and nausea) and adjust their intake to reduce these effects or as tolerance develops allowing them to continue to smoke. Interestingly, DiFranza and colleagues [16] noted that among first-time users (primarily young adolescents), throat irritation with the first puff is a predictor of reduced cigarette use whereas relaxation, dizziness, and nausea predict subsequent cigarette use disorder (see also [17, 18]). In a self-report of the effects of mescaline, the user described vivid hallucinations but noted aversive side effects such as nausea and dizziness that led to speculation that the drug would not likely become popular given that such side effects would spoil the generally positive effects of the drug [14]. One also sees these aversive effects with injected heroin as the drug has been reported to induce an orgasmic rush that is often accompanied by nausea, retching, and vomiting. These aversive side effects diminish with repeated dosing [19]. Effects of caffeine have also been reported to reflect the interaction of its rewarding and aversive effects, and this interaction appears to be dose-dependent. For example, in an assessment of intake and reactions to varying doses of caffeine, low doses (e.g., 100 mg) were found to be positively rewarding to all subjects (with none reporting any negative effects). With increases in dose, a general preference for the drug decreased as specific aversive or unwanted effects such as jitteriness and nervousness appeared [14]. Similar dose-related effects have been reported with phencyclidine (PCP). For example, at low doses, PCP produces a rewarding effect that is often accompanied by a range of aversive effects, e.g., thought disturbances, as well as violent behavior. With even greater doses, the aversive and unpleasant side effects such as panic, fear paranoia, incoherent speech, and bizarre behaviors become more intense that may dimmish the likelihood of further intake (see also [15, 20]).

Work with alcohol further demonstrates the aversive effects of drugs and how these effects modulate or impact drug intake. For example, the mutation in the gene coding for the enzyme aldehyde dehydrogenase (from the typical isozyme ACDH2 to the less efficient isozyme ACDH2*2; found predominately in East Asian populations) results in the reduced ability to metabolize acetaldehyde, a metabolite of alcohol [21]. Acetaldehyde has been reported to produce a variety of adverse reactions, e.g., flushing of the face, headaches, and heart palpitations (with the severity of these reactions greater in individuals homozygous for the ACDH2*2 gene), and appears to be protective against further alcohol intake among those with the gene for this enzyme (see [22]; for evidence of acetaldehyde's rewarding and motivating effects, see [23]. The use of the drug disulfiram, a drug that blocks the metabolism of acetaldehyde, in the treatment of alcoholism is based on this same principle.

In an assessment of patterns of alcohol intake in humans, Baker and Cannon [24] noted that approximately 45% of individuals hospitalized for the treatment of alcoholism reported aversions to the flavor of specific alcohol preparations most of which were acquired as a function of overconsumption during early adolescence. That is, becoming sick with their initial alcohol experience limited subsequent consumption of those specific beverages. Similarly, based on a survey of taste aversions in humans, Logue et al. [25] reported upwards of 25% of 517 individuals who answered the survey indicated aversions to alcohol that were associated with earlier patterns of alcohol consumption. It is interesting in this context that one chemical treatment of alcoholism utilizes aversion therapy in which alcohol consumption is associated with an injection of a nauseant drug that induced aversions to the taste of alcohol ([26–28]; for reviews, [29, 30]). Further (and along the lines noted above with genetic mutations), individuals appear to be differentially sensitive to these aversive effects of alcohol evidencing another genetic vulnerability, in this case toward greater consumption in those individuals less affected by alcohol's aversive effects. Importantly, these vulnerabilities appear to interact with experience as well given that individuals who do not initially experience aversive effects seem to be protected from subsequent aversive reactions (either through tolerance to alcohol or the added rewarding effects of alcohol in ameliorating withdrawal symptoms; see [24]).

These data from clinical populations illustrate that drugs of abuse are complex pharmacological agents that possess multiple stimulus effects, with the rewarding effects increasing vulnerability to initial use and subsequent abuse and the aversive effects limiting intake. Such effects can occur at the same dose; some are dose-dependent. Some effects appear to be impacted by genetic vulnerabilities; some are affected by experience. Independent of the drug and the factors that modulate its effects, what is clear is that individuals weigh the balance of these effects and intake is either adjusted or continued with the anticipation that the aversive effects will be lessened with use (tolerance) or will become less salient as the rewarding effects increase (sensitization).

5. Preclinical Evidence of the Aversive Effects of Drugs of Abuse: Taste Aversions

Work with preclinical populations also reports evidence of aversive effects of drugs of abuse. While there is considerable support for such effects in preclinical literature, the roots of this evidence are in toxicology [31]. In fact, work demonstrating such effects came from investigations related to military applications during World War II, i.e., the effects of toxins on rodent infestations (see [32]) and the effects of radiation exposure on biological systems (see [33]; for a review, see [34]). Initial field trials on rodent management with baits (i.e., a poison mixed with food base) presented a

major difficulty, as rats exhibit a neophobic response toward novel foods and rarely sample enough of the bait to ingest a lethal amount of the poison. In early studies of this phenomenon (bait-shyness), Rzóska [32] fed rats a food base laced with poison and noted that rats that had initially accepted the poisoned bait avoided the same bait in successive trials, but when a new base laced with the same poison was offered, they readily consumed the new bait. In speculating on these empirical findings, i.e., refusal of identical poisoned bait and acceptance of experienced poison in the new base, Rzóska concluded that the rats associated the food base, rather than the poison itself, with the illness experienced following ingestion of the bait so they avoided the same food on subsequent trials.

In the early 1950s, this phenomenon of associative learning between a novel taste and illness was further demonstrated with the effects of radiation by Garcia and his colleagues who observed that rats given water in plastic bottles during radiation exposure subsequently avoided drinking water from those plastic bottles. Importantly, the same rats would drink the water provided in glass bottles, suggesting that the plastic bottle gave the water a unique taste that was associated with the effects of radiation (for a history of Garcia's early work with radiation, see [34]). In subsequent studies, Garcia and colleagues [33] tested the basis of these aversions by giving rats a novel saccharin solution to drink during radiation exposure and reported that those rats strongly suppressed consumption of the saccharin solution after a single pairing of saccharin with radiation compared to the control group that was not exposed to radiation following intake of the saccharin. Garcia et al. concluded that the aversive effects of radiation conditioned an aversion to the radiation-paired flavor (see Figure 2). This initial report demonstrated the fast and robust nature of conditioned taste aversions (CTAs) as a form of classical conditioning, wherein learning occurred with a single pairing; the aversion was dose-dependent (30 vs. 57 roentgen (r)) and evident for over 30 days post conditioning despite the fact that animals were given continuous access to the initially preferred saccharin solution and water during this period. Such aversions have been reported to be maintained with a year (53 weeks) intervening between its acquisition and eventual test [35].

Subsequently, Garcia et al. [36] demonstrated that an aversion to saccharin was acquired with an interstimulus interval as long as 75 min (i.e., when the delay between consumption and radiation was 75 min). In a separate study published the same year, taste aversion learning appeared selective to gustatory stimuli, wherein rats selectively associated saccharin with radiation but audiovisual cues with foot shock [37]. These unique conditions under which CTA was acquired, that is, learning with one trial, over long delays and relatively selective to tastes, led to the reconceptualization of the role of evolution in shaping behavior and learning. It seems plausible that natural selection favored organisms able to quickly learn the taste-illness association. Given that aversive outcomes are likely to occur after some delay as the natural function of digestion, the ability to learn a taste-illness association over long delays prevents repeated consumption of toxic foods. Such adaptive specialization for survival also extended to the selective nature of CTA learning that prevents irrelevant stimuli (e.g., external cues) from interfering with the learning of a taste-illness association [38–40].

6. Taste Aversions as an Index of Toxicity

Although the initial investigations into CTAs primarily focused on their empirical assessments and theoretical implications, subsequent research in this area shifted to explore the conditions under which CTA learning can be acquired, effects of various manipulations on its expression, and issues of mechanisms and applications. An important extension involved the use of CTA preparation as a tool to detect and characterize the behavioral and physiological effects of a toxin [31]. Empirically, the application of CTA as an index of toxicity is supported by the evidence that a wide range of classical toxins that were characterized by other behavioral and pharmacological tests could also condition taste aversions under various experimental conditions (for a review, see [31]). For example, Nachman and Hartley [41] demonstrated that various rodenticides highly toxic to rats (i.e., copper sulfate, red squill, and sodium fluoroacetate) produced strong taste aversions often with only a single pairing of a novel taste with the compound. In addition to being rapidly learned, CTAs appeared relatively sensitive to detecting the aversive effects of drugs relative to other indices of toxicity. For example, trimethyltin, a known neurotoxicant that causes specific damage to the hippocampus, induces taste aversions [42, 43]. In more traditional behavioral assays, a single administration of trimethyltin disrupts hippocampus-dependent performance as measured in a number of tasks, e.g., Hebb-Williams maze, radial-arm maze, and differential reinforcement of low rates of responding (DRL). Interestingly, the dose of trimethyltin needed to condition a taste aversion is 500% less than that required to produce effects in other behavioral indices of toxicity [42, 43]. These dose-response comparisons substantiate the taste aversion design as a sensitive index in detecting toxicity, as compounds that support taste aversions generally do so at lower doses than are necessary to produce effects in more traditional assessments of toxicity. From a theoretical perspective, the sensitivity of the taste aversion design appears to be a natural extension of the concept of adaptive specialization in normal consummatory behavior. An organism that can learn the toxic potential of its food source is likely to quickly avoid subsequent toxicosis and reduce the possibility of ingesting the fatal dose of the toxin (see above). Research investigating other compounds with toxic and adverse effects within the CTA preparation steadily increased throughout the 1970s with several well-known toxins such as barium sulfate, cyanide, red squill, strychnine sulfate, and sodium fluoride reportedly producing CTAs (for a more comprehensive list of compounds with toxic and adverse effects that induce CTAs, see Table 1). Although most classical toxins (and neurotoxins) reliably condition taste aversion, several compounds with known toxicity have been reported to be ineffective. While such caveats clearly suggest a limitation of the CTA preparation as an index of toxicity, alternative interpretations have been raised in

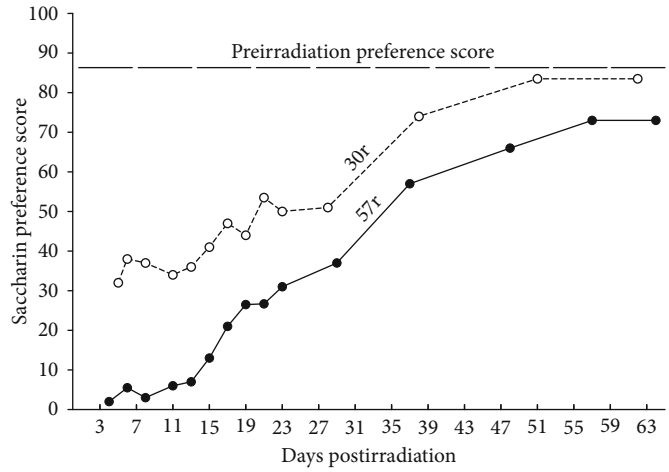

Figure 2: Median saccharin preference scores for animals previously given saccharin access during radiation exposure. Redrawn from Garcia et al. [33].

relation to specific procedures in assaying taste aversions that might account for these failures (see [31] for a discussion of the basis for the failure of known toxins to condition taste aversions and procedural variations of the CTA preparation that could increase the efficacy of those compounds to induce CTA).

There are certainly other behavioral assays for the aversive effects of drugs than taste aversion learning, e.g., suppression of normal regulatory behavior (food and water intake) and disruptions of scheduled-controlled responding, activity, learning and memory, and hedonic shifts (see Riley and Tuck, 1985 [31]). One assay very related procedurally to conditioned taste aversions is the conditioned place aversion (CPA) preparation in which specific environments (or contextual cues) are associated with drug injection. In this preparation, animals avoid or spend less time in the drug-paired environment than in one that is paired with the drug vehicle. Although this procedure is often used to assess the aversive effects of a drug, it should be noted that when direct comparisons have been made between the taste and place aversion designs, aversions are generally more rapidly acquired and more strongly evident in taste (than place) conditioning (for a direct comparison between LiCl-induced taste and place aversions and a review of other drug comparisons in these two designs, see [99]). These differences between the taste and place conditioning procedures in such assessments are likely a function of the relatively greater associability of taste (over place) in the conditioning of aversive effects (see [38–40]). It is important to note that the majority of drugs of abuse that reliably induce taste aversions fail to induce a place aversion (in fact, generally inducing a place preference; see below) or produce a CPA under specific parametric conditions (high doses, without a drug history, and time of injection relative to placement in chamber) or with specific sex, age group, or species (for a discussion, see [99]). Given that taste aversion conditioning has been more extensively examined as a behavioral assay of the aversive effects of drugs and does so with greater sensitivity and generality, the present review focuses primarily on conditioned taste aversion learning in our analysis.

7. Conditioned Taste Aversions Induced by Drugs of Abuse

Although the initial work on the conditions supporting taste aversion learning assessed compounds with adverse or toxic effects as potential aversive stimuli, by the early 1970s, a host of other compounds were being investigated, some of which included drugs of abuse. For example, Lester et al. [100] assessed taste aversion learning with ethanol in which male Wistar rats were given 10 min access to a saccharin solution that was then followed by administration of ethanol (at various concentrations and doses and by different routes of administration). Under these parametric conditions and with only a single conditioning trial, ethanol induced significant suppression of saccharin consumption, and as reported with work with known toxins [34], the degree of the aversions induced was dose-, concentration-, and route-dependent. Importantly, control subjects receiving the same saccharin solution paired with injections of the ethanol vehicle readily consumed it, indicating that the suppression evident in the ethanol-treated animals was a function of the association of saccharin with ethanol.

The following year, Cappell and LeBlanc [101] assessed the aversive effects of several other drugs of abuse, specifically mescaline and d-amphetamine. In the assessment with mescaline, male Wistar rats were given access to a novel saccharin solution and injected intraperitoneally with 0 (vehicle), 20, 36, or 62.4 mg/kg mescaline hydrochloride, and after, only a single pairing saccharin consumption was significantly suppressed in all groups injected with mescaline (maximum suppression at 36 mg/kg). In other groups of rats, amphetamine (administered intraperitoneally at 0, 2, 4, and 8 mg/kg) was given following saccharin consumption, and significant aversions were again evident at all doses (maximum suppression at 2 mg/kg). Control subjects consumed at high levels following the saccharin-saline pairing. Subsequent work by Cappell and his colleagues [102] replicated Lester et al. [100] by reporting dose-dependent ethanol-induced CTAs in male Wistar rats and extended the classes of drugs that were effective in inducing aversions

Table 1: Compounds with adverse or toxic effects effective in producing CTAs.

Compound	Reference
α-Naphthylthiourea (rodenticide)	Rzóska, 1954a [32]
1,1,2-Trichloroethane (carcinogen)	Kallman et al., 1983 [44]
1,2-Dicholoethane (probable carcinogen)	Kallman et al., 1983 [44]
1,2-Dichloroethylene (health hazard)	Kallman et al., 1983 [44]
2,3,5-Trimethylphenyl methyl carbamate (neurotoxin)	Nicolaus, 1987 [45]
2,4,5-Trichlorophenoxyacetic acid (herbicide)	Sjödén and Archer, 1977 [46]
6-Formylindolo (3,2-b) carbazole (FICZ) (carcinogen)	Mahiout and Pohjanvirta, 2016 [47]
Acetaldehyde (primary metabolite of ethanol)	Brown et al., 1978 [48]
Acetoxycycloheximide (protein synthesis inhibitor)	Ungerer et al., 1975 [49]
Acrylamide (neurotoxin)	Anderson et al., 1982 [50]
Adriamycin (gastrointestinal tract toxin)	Bernstein et al., 1980 [51]
Aflatoxin B1 (toxic to liver and kidney)	Rappold et al., 1984 [52]
Alloxan monohydrate (diabetogenic agent)	Brookshire et al., 1972 [53]
Arsenic (rodenticide)	Rzóska, 1954a [32]
Atrazine (chlorotriazine herbicide)	Hotchkiss et al., 2012 [54]
barium carbonate (rodenticide)	Rzóska, 1954a [32]
Baygon (insecticide)	Ebeling, 1969 [55]
Benzo[α]pyrene (BaP) (dioxins)	Mahiout and Pohjanvirta, 2016 [47]
Boric acid (pesticide)	Ebeling, 1969 [55]
Bufotoxin (neurotoxin)	Ward-Fear et al., 2016 [56]
Cadmium (toxic metal)	Wellman et al., 1984 [57]
Carbaryl (insecticide)	MacPhail and Leander, 1980 [58]
Chloral hydrate (potent sedative)	Kallman et al., 1983 [44]
Chlordimeform (insecticide)	Landauer et al., 1984 [59]
Cisplatin (cytotoxin)	Revusky and Reilly, 1989 [60]
Clorgyline (neurotoxin)	Buresová and Bures, 1987 [61]
Cobalt chloride (toxic to organs)	Wellman et al., 1984 [57]
Cobra venom (neurotoxin)	Islam, 1980 [62]
Copper sulfate (pesticide)	Nachman and Hartley, 1975 [41]
Cyanide (cytotoxin)	O'Connor and Matthews, 1995 [63]
Cycloheximide (protein synthesis inhibitor)	Booth and Simson, 1973 [64]
Cyclophosphamide (gastrointestinal tract toxin)	Dragoin et al., 1971 [65]
Cytoxan (cytotoxin)	Bernstein et al., 1980 [51]
Dactinomycin (cytotoxin)	Revusky and Martin, 1988 [66]
Denatonium benzoate (rodenticide)	El Hani et al., 1998 [67]
Doxorubicin (cytotoxin)	Revusky and Martin, 1988 [66]
Emetine hydrochloride (emetic)	Cannon and Baker, 1981 [68]
Ferric nitrilotriacetate (Fe-NTA) (renal carcinogen)	Irie et al., 2000 [69]
Formalin (systemic poison)	Stricker and Wilson,1970 [70]
Ipecacuanha (emetic)	Rudd et al., 1998 [71]
Krait venom (neurotoxin)	Islam, 1980 [62]
Lead (toxic metal)	Leander and Gau, 1980 [72]
Lipopolysaccharide (endotoxin)	Exton et al., 1995 [73]
Mechlorethamine (vesicant)	Revusky and Martin, 1988 [66]
Mercuric chloride (cumulative poison)	Klein et al., 1974 [74]
Methyl bromide vapor (cumulative poison)	Miyagawa, 1982 [75]
Methylmercury (neurotoxin)	Levine, 1978 [76]
Methiocarb (pesticide)	Mason and Reidinger, 1982 [77]

Table 1: Continued.

Compound	Reference
Metrazol (convulsant)	Millner and Palfai, 1975 [78]
Mesurol (pesticide)	Gustavson et al., 1982 [79]
Sodium fluoroacetate (rodenticide)	Nachman and Hartley, 1975 [41]
n-Butyraldoxime (aldehyde dehydrogenase inhibitor)	Nachman et al., 1970 [80]
N-N-Ethyl-2-bromobenzylamine (neurotoxin)	Archer et al., 1983 [81]
Ochratoxin (mycotoxin)	Clark and Wellman, 1989 [82]
Ozone (toxic to lung)	MacPhail and Peele, 1992 [83]
Paraquat (herbicide)	Dey et al., 1987 [84]
p-Chlorophenylalanine (neurotoxin)	Nachman et al., 1970 [80]
Phenylthiocarbamide (neurotoxin)	St. John et al., 2005 [85]
Picrotoxin (GABA receptor inhibitor)	Chester and Cunningham, 1999 [86]
Red squill (rodenticide)	Rzóska, 1954a [32]
Sarin (neurotoxin)	Landauer and Romano, 1984 [87]
Scorpion venom (neurotoxin)	Islam, 1980 [62]
Sodium cyanide (rodenticide)	Nachman and Hartley, 1975 [41]
Soman (neurotoxin)	Romano et al., 1985 [88]
Staphylococcal enterotoxin B (exotoxin)	Kusnecov et al., 1999 [89]
Strychnine sulfate (rodenticide)	Howard et al., 1968 [90]
T-2 toxin (mycotoxin)	Wellman et al., 1989 [91]
Thallium sulfate (rodenticide)	Nachman and Hartley, 1975 [41]
Thiabendazole (pesticide)	Gustavson et al., 1983 [92]
Thiram (fungicide)	Tobajas et al., 2019 [93]
Tumour necrosis factor α (cytokines)	Goehler et al., 1995 [94]
Trichloroethylene (carcinogen)	Kallman et al., 1983 [44]
Trichloromethane (neurotoxin)	Balster and Borsellca, 1982 [95]
Triethyltin (neurotoxin)	MacPhail, 1982 [42]
Trimethyltin (neurotoxin)	MacPhail, 1982 [42]
Triphenyltin (fungicide)	MacPhail and Peele, 1992 [83]
Toluene (systemic toxin)	Miyagawa et al., 1984 [96]
Viper venom (hemotoxic)	Islam et al., 1982 [62]
Vomitoxin (mycotoxin)	Clark et al., 1987 [97]
Xylene (systemic toxin)	MacPhail and Peele, 1992 [83]
Ziram (fungicide)	Baker et al., 2005 [98]

to morphine and chlordiazepoxide (3, 6, and 9 mg/kg; intraperitoneal). Importantly, this work revealed that while aversions were dose-dependent, the strength of the aversions and the doses at which significant aversions were evident varied across drugs, suggesting drug-specific aversive effects. Concurrent with (and subsequent to) these initial investigations, a wide range of drugs of abuse known for their ability to support self-administration [103] induced taste aversions as well (see Table 2 for a comprehensive list of various drugs of abuse effective in inducing conditioned taste aversions), suggesting that such drugs produce a number of stimulus effects (both rewarding and aversive).

Almost immediately upon these various demonstrations, a question was raised as to how drugs that were readily self-administered could also be aversive (as indexed by taste aversion learning; see [127, 128], i.e., the two opposing stimulus effects seemed paradoxical. At the outset, it was recognized that these two effects, i.e., rewarding and aversive, were generally assessed via different procedures (and generally in different laboratories). As such, demonstrations of these multiple stimulus effects may be a function of the specific procedure under which they were assessed and not necessarily paradoxical. As examples of these differences, Cappell and his colleagues [127] noted that with work assessing the self-administration of drugs, the drug is generally administered intravenously and under the control of the subject (for a discussion of alternatives, see [10, 129]), whereas in typical taste aversion studies, subjects are given the drug intraperitoneally, subcutaneously, or orally ([34, 130, 131]; though see [132–136] for evidence of taste aversions induced by intravenously delivered drug) and at the control of the experimenter and not the subject (though

Table 2: Drugs of abuse that are effective in producing a CTA. Each drug has the reference for one of the initial studies examining that specific drug.

Compound	Reference
α-Pyrrolidinopentiophenone (α-PVP) (synthetic cathinone; CNS stimulant)	Nelson et al., 2017 [104]
Δ^9-Tetrahydrocannabinol (Δ^9-THC) (cannabinoid)	Elsmore and Fletcher, 1972 [105]
3,4-Methylenedioxymethamphetamine (MDMA) (hallucinogen)	Lin et al., 1993 [106]
3,4-Methylenedioxypyrovalerone (MDPV) (synthetic cathinone; CNS stimulant)	King et al., 2014 [107]
Amobarbital (CNS depressant)	Vogel and Nathan, 1975 [108]
Amphetamine (CNS stimulant)	Berger, 1972 [109]
Barbital (CNS depressant)	Jolicoeur et al., 1977 [110]
Caffeine (CNS stimulant)	Dickens and Trethowan, 1971 [111]
Cathinone (CNS stimulant)	Goudie and Newton, 1985 [112]
Cannabidiol (CBD) (cannabinoid)	Corcoran et al., 1974 [113]
Cannabigerol (CBG) (cannabinoid)	Corcoran et al., 1974 [113]
Cocaine (CNS stimulant)	Goudie et al., 1978 [114]
CP 55,940 (synthetic cannabinoid)	McGregor et al., 1996 [115]
d-Amphetamine (CNS stimulant)	Cappell and LeBlanc, 1971 [101]
Diazepam (CNS depressant)	Jolicoeur et al., 1977 [110]
Ethanol (CNS depressant)	Lester et al., 1970 [100]
Ethanol (CNS depressant)+cocaine (CNS stimulant)	Busse et al., 2005 [116]
Flurazepam (CNS depressant)	Vogel and Nathan, 1975 [108]
Heroin (analgesic)	Grigson et al., 2000 [117]
Heroin (analgesic)+cocaine (CNS stimulant)	Riley et al., 2019 [118]
Hexobarbital (CNS depressant)	Vogel and Nathan, 1975 [108]
Ketamine (hallucinogen)	Etscorn and Parson, 1979 [119]
l-Amphetamine (CNS stimulant)	Carey and Goodall, 1974 [120]
Lysergic acid diethylamide (LSD) (hallucinogen)	Parker, 1996 [121]
Methamphetamine (CNS stimulant)	Martin and Ellinwood, 1973 [122]
Mescaline (hallucinogen)	Cappell and LeBlanc, 1971 [101]
Methaqualone (sedative hypnotic)	Vogel and Nathan, 1975 [108]
Methyprylon (sedative hypnotic)	Jolicoeur et al., 1977 [110]
Methylone (synthetic cathinone; CNS stimulant)	Manke et al., 2021 [123]
Methylphenidate (CNS stimulant)	Riley and Zellner, 1978 [124]
Morphine (analgesic)	Cappell et al., 1973 [102]
Nicotine (CNS stimulant)	Etscorn, 1980 [125]
Pentobarbital (CNS depressant)	Buresova and Bures, 1980 [126]
Phencyclidine (PCP) (hallucinogen)	Etscorn and Parson, 1979 [119]
Phenobarbital (CNS depressant)	Vogel and Nathan, 1975 [108]

see [117, 137–139] for demonstrations of aversions induced when the drug was self-administered and/or under the control of the subject).

In addition to these basic procedural differences in drug self-administration vs. taste aversion learning, such demonstrations of the rewarding and aversive effects were often assessed under different parametric conditions, e.g., with different sexes, at different ages, in different strains, at different doses, and under different deprivation schedules, following acute and chronic exposure. The possibility that demonstrations of reward and aversion were a function of simple parametric differences between such demonstrations was soon dismissed with reports that the aversive and rewarding effects of many drugs of abuse could be seen in a design that concurrently assessed these effects which assured that the parametric conditions under which any effects were tested were identical. For example, Wise et al. [137] gave rats access to saccharin and then immediately allowed them to self-administer apomorphine (0.5 mg/kg per infusion; all subjects had previous experience with the intravenous self-administration of amphetamine). On the subsequent exposure to saccharin, 10 of the 11 subjects trained and tested displayed aversions to the apomorphine-associated saccharin solution with the degree of the aversion directly related to the amount of apomorphine self-administered during the initial training session. Thus, both the rewarding (self-

administration) and aversive (CTA) effects of apomorphine were evident under the same parametric conditions (and at comparable doses), suggesting multiple (and opposing) stimulus effects of the drug. In a related study, White et al. [140] reported that rats injected with morphine after running down a straight alley to obtain food ran faster to obtain the food (reward) but failed to consume it (aversion), again revealing dual (and concurrent) effects of a drug, in this case morphine. Interestingly, animals given the emetic LiCl under the same conditions displayed reduced running speed to obtain the food and failed to eat the food, as well. In related work, Ettenberg and Geist [138, 139] have reported both positive and negative effects of cocaine in a runway model. In this design, animals are allowed to run down a straight alley for an intravenous injection of cocaine. After several such trials, the latency to leave the start box decreased (indicative of cocaine's rewarding effects) and the running time to enter the goal box increased as animals began to retreat from the goal box with further training (indicative of cocaine's aversive effects). Ettenberg et al. suggested that the immediate actions of cocaine were rewarding (decreasing response latencies) that were quickly followed by an opponent process crash that resulted in an approach/avoidance reaction and increased running time as animals retreated from (avoided) the goal box that was associated with cocaine (see also [141]; for evidence of this time-dependent opponent process of reward/aversions, see [142]).

8. Combined Taste Aversion/Place Preference Procedure

Shortly after the demonstrations by Wise et al. [137] and White et al. [140], Reicher and Holman [143] used a different procedure to assess the aversive and rewarding effects of amphetamine. Specifically, this group used a combined conditioned taste aversion and conditioned place preference (CTA/CPP) design in which they gave female Sprague-Dawley rats an intraperitoneal injection of amphetamine (1.43 mg/kg) and placed them on one side of a two-compartment shuttle box during which they had access to a novel-flavored solution (banana or almond). On the next day, the animals were injected with the amphetamine vehicle and placed on the opposite side of the shuttle box with access to the other novel solution (almond or banana). Six such alternating trials were given followed by tests for side and flavor preferences. Under these conditions, amphetamine induced significant taste aversions and place preferences, again demonstrating both aversive and rewarding effects of the same drug and under identical parametric conditions. Subsequent to the initial work by Reicher and Holman, a variety of drugs have now been shown to support both effects in the combined CTA/CPP procedure including 3,4-methylenedioxypyrovalerone (MDPV) [144], α-pyrrolidinopentiophenone (α-PVP) [104, 145, 146], nicotine [147], amphetamine [148–150], morphine [150–157], cocaine [158–160], alcohol [161], and caffeine [162]; for several drugs, e.g., ethanol [163] and Δ^9-tetrahydrocannabinol (THC) ([164, 165], both taste and place aversions were reported with the combined design (for reviews, see [166, 167]).

The advantage of using a combined procedure to examine a drug's aversive and rewarding effects in the same animal is that it addresses the concern that the two effects are simply a function of different experimental conditions under which they are tested (see above). While this is true, the combined procedure as generally used does train the animal in a serial manner, i.e., the animal is given access to some novel solution, e.g., saccharin, injected with the drug and then put on one side of the place preference apparatus. Under such conditions, one could argue that acquisition of the CTA itself or the conditions under which the CTA is generally trained and tested, i.e., water deprivation, could impact the acquisition (or display) of the place preference. As such, the measure of reward in terms of place preference conditioning within the combined design might differ from what one would see if place preferences were assessed separately.

Although there are many studies using the combined design (see above), there are only a few that have addressed this potential confound. For example, in one such study, animals were given morphine-induced taste aversion training, and once aversions were established, the same animals were assessed for place preference conditioning with morphine. Place preferences were then compared between groups that had the aversion history vs. those that had been given control injections during the taste aversion training, i.e., control subjects with no pairings of the taste with morphine. Under these conditions, there were no differences between animals with or without the taste aversion history, as morphine-induced place preferences were similar for both groups [153]. These results suggest that having a history in which a specific drug induced an aversion had no effect on its ability to induce a place preference for that same drug. While this addresses the effects of an aversion history on place preference conditioning, it does not address the possibility that the procedures used in the combined design to induce an aversion might impact the acquisition of the place preference. One factor that might impact such learning would be water deprivation (or its associated stress). In the combined design, animals are generally water deprived to encourage consumption, but it is present as well during the place preference assessment, a condition not typically used in independent assessments of place preference conditioning. In a recent study, Dannenhoffer and Spear [147] examined the combined CTA/CPP procedure with nicotine in nondeprived adolescent and adult rats. To induce drinking in these nondeprived animals, a highly palatable sucrose/saccharin solution was given after which the animals were injected with nicotine and placed on one side of a place preference chamber. As expected, nicotine induced significant taste aversions and place preferences, and importantly, place preferences (and taste aversions) were similar to those reported in independent assessments of each. Further, adolescents were more sensitive to the rewarding effects of nicotine (and less sensitive to its aversive effects) relative to adults (for comparison, see [168] who reported these same relative sensitivities of CPP and CTA when separately assessed).

Other comparisons across studies have shown that place preference conditioning is comparable when assessed in a combined CTA/CPP design or as an independent CPP and that manipulations in either design impact place preference conditioning similarly (see [152, 157] for assessments of the combined CTA/CPP assay with morphine compared to [169, 170] for assessments of morphine using only CPP; for a similar comparison with MDPV, see [171] compared to [172]; for α-PVP, see [104] compared to [173, 174]; and for caffeine, see [162] compared to [175]).

9. Implications of the Aversive and Rewarding Effects of Drugs

The fact that drugs have both rewarding and aversive effects raises an interesting issue regarding their possible role in drug intake. Specifically, the balance of these two effects may be important in the likelihood of a drug's initial use and its continued (regulated) intake (see Figure 3). Further, we are suggesting that it is this very balance that predicts the abuse vulnerability of a specific drug [20, 137, 141, 166, 176–179]. If the rewarding effects of the drug (at any given dose) are greater than its aversive effects at that same dose, the abuse vulnerability of this drug might be predicted to be high as intake may increase with its overall greater affective value. If at the outset the drug's aversive effects (at any given dose) exceed its rewarding effects at that same dose, it might be expected that drug intake would not continue and the drug would have limited abuse potential. It is important to note that this balance is not fixed for any specific drug as a wide range of factors have been reported to affect both its rewarding and aversive effects (see below) which, in turn, can shift the affective balance. It is also important to note that the nature of the drug's rewarding effects changes with more frequent and chronic use (from positive associated with acute and regulated intake to negative associated with dysregulated intake and the onset of dependence, anhedonia, and withdrawal (see [4–7]). Although under these latter conditions, drug intake may still be a function of the balance of reward and aversion; the very nature of anhedonia may weaken the relative contribution of the drug's aversive effects as individuals use the drug for relief from withdrawal despite their aversive effects which would normally limit intake.

10. Dissociation between the Aversive and Rewarding Effects of Drugs

The fact that these two stimulus effects are evident in the same animals and often under similar parametric conditions supports the position that a drug has multiple affective properties. Interestingly, it appears that these two effects can be dissociated. For example, Verendeev and Riley [150] (see also [180]) have reported that animals trained in a combined CTA/CPP procedure with morphine (5 or 10 mg/kg, intraperitoneal) or amphetamine (3 or 5 mg/kg, intraperitoneal) acquired taste aversions as well as place preferences; however, there was no relationship between the strength of taste aversion and place preference conditioning for either drug.

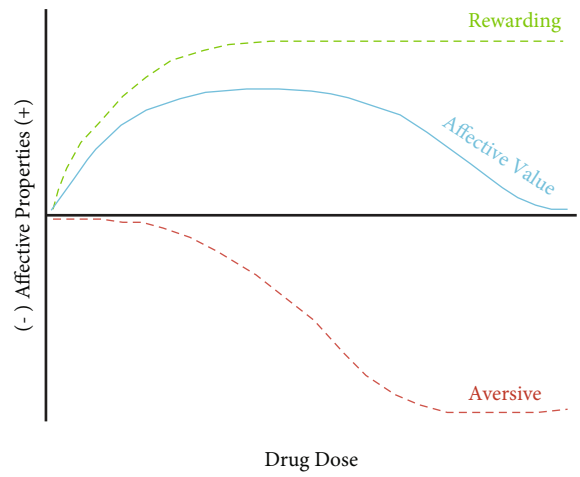

Figure 3: A hypothetical model of the aversive and rewarding effects of a drug and their potential interaction to impact its self-administration (which is a function of the overall affective response to the drug). The drug produces both aversive and rewarding effects in a dose-dependent manner. As illustrated in this specific example, the drug's rewarding effects are produced at lower doses that increase the drug's overall affective property that, in turn, drives the drug's intake. With increases in the dose, the drug's rewarding effects asymptote while the drug's aversive effects increase, reducing the overall affective value of the drug and decreasing the drug's self-administration. In this model, the drug's rewarding effects are assumed to initiate and maintain drug intake (at least under acute conditions) while its aversive effects limit it. The nature of such an interaction is not static and depends upon a host of factors (see Sections 11 and 12). Further, the relative contributions of the aversive effects in limiting intake change as drug intake go from regulated to dysregulated given the change in the reward valence from positive to negative. Created with BioRender.com.

Interestingly, animals that acquired strong morphine-induced taste aversions were just as likely to display weak or strong morphine-induced place preferences. Similarly, animals that acquired weak morphine-induced taste aversions were just as likely to display weak or strong place preferences (for related findings with serial conditioning of CTAs and CPPs, see [180]). That is, there was no relation between the two measures. The same pattern emerged with amphetamine. King et al. [171] have also reported similar independence with the synthetic cathinone, MDPV. In this report, males and females both acquired dose-dependent taste aversions with males displaying greater aversions than females. On the other hand, while both sexes acquired MDPV-induced place preferences, these were independent of sex and correlational analysis between the degree of taste aversions and place preferences did not reveal a consistent relationship between the two measures (see [145] for related findings with the synthetic cathinone a-PVP in which both males and females displayed significant and dose-dependent CTAs (M > F), but only males displayed significant place preferences).

The apparent dissociation of taste aversions and place preferences as measured in the combined CTA/CPP design argues that these two effects occur concurrently but are not

related. The fact that the aversive effects of the drug are associated with taste (CTA) while the rewarding effects are associated with a specific place (CPP) is likely a function of the relative selectivity of taste and environment conditioning with these specific affective properties (see [33, 38, 40]; for a review, see [34]). Although apparently dissociable, any attempt to relate these behaviors in a correlational analysis should be made cautiously, given that CTA and CPP procedures may differ in their relative sensitivity as measures of the aversive and rewarding effects of drugs, respectively. For example, CTA may be less sensitive as a measure of the aversive effects of a drug than CPP is as a measure of its rewarding effects, and the drug's aversive effects may not be accurately reflected in the expression of taste aversions. Conversely, CPP may be less sensitive as a measure of the rewarding effects of a drug than CTA is as a measure of its aversive effects. That is, the drug's rewarding effects may not be accurately reflected in the expression of place preferences. As such, any attempt to relate these behaviors in a correlational analysis should be made cautiously. Interestingly, under conditions where taste aversions and place preferences have been analyzed in individual subjects most sensitive to either aversive (high CTA) or rewarding (high CPP) effects of drugs, there still is no consistent relationship between the ability of either drug to produce these effects (see [144, 150]), supporting the ability of the aversive and rewarding effects to cooccur and at the same time being dissociable—a position consistent with the drugs having cooccurring, but unrelated, effects.

Such a position is supported by other assessments of drug-induced taste aversions and place preferences that have found similar dissociations between the two effects in a number of studies evaluating the impact of a variety of manipulations on their acquisition and display, e.g., age [147, 168, 181], sex [145], strain [181–183], drug history [146, 152, 182, 184], drug interactions [116], genetic knockouts/knockins [185, 186], lesions [133, 187], state dependency [188], role of DARRP-32 [189], effects of LPS [190], effects of ^{56}Fe particles [191], neuroanatomical and neurochemical mediation [157, 192, 193], and receptor subtype [186, 194]. For these assessments, CTA and CPP were differentially affected, suggesting that the two were unrelated, i.e., if related one would expect that the two effects would be similarly impacted. In the above evaluations, CPP could be seen with no evidence of a CTA (and vice versa) or CPP could be increased and CTA decreased (or vice versa) by chemical and neuroanatomical challenges. Although these demonstrations were generally made with separate analyses of reward and aversion, i.e., using the CTA (or conditioned place aversion) design to assess the drug's aversive effect and the CPP design to assess reward, some were made with the combined design demonstrating again that the dissociations were not a simple function of parametric conditions.

One specific manipulation that deserves special attention is that of dose. As noted in the clinical literature (see above), the rewarding and aversive effects of some drugs were reported to be dose-dependent, specifically rewarding at low doses and aversive at higher ones. While there is evidence of dissociations between place preferences and taste aversions based on studies for which the doses supporting the two effects differ (see [161, 162, 164, 165, 195]), it is important to note that under most of the assessments cited above reporting the dissociation of CTAs and CPPs, comparable doses were administered, yet the two indices of the drug's affective properties were still differentially affected by various manipulations [116, 145–147, 152, 159, 168, 181, 182, 188, 191], i.e., the different effects reported for CTA and CPP were not simply due to animals being tested under different doses in the two designs.

11. Nature of the Aversive Effects of Drugs

The present review has discussed conditioned taste aversions in the context of their origins and extensions. As such, it has used the animal's suppression of consumption of a specific taste following its pairing with either a toxin or drug of abuse to be a function of the compound's aversive effects that become conditioned to the taste itself. Noting that drugs of abuse have aversive effects that may limit (or modify) their intake suggests that these effects may be important in regulated drug intake; however, such a position does not indicate their nature which has been somewhat elusive over the history of the phenomenon of taste aversion learning [34]. The present review is somewhat neutral on this issue, but suffice it to say, the literature has had much to discuss (and debate) regarding the basis of taste aversion conditioning. At the outset of work on this phenomenon [33, 196], the avoidance was thought to be a function of conditioning of the radiation-induced gastrointestinal effects, e.g., sickness. The resulting avoidance was a reflection of conditioned sickness/malaise itself, i.e., a conditioned aversion to that taste [38]. As reported by Garcia and Kimeldorf [197], radiation localized to the abdomen induced significant taste aversions at doses that had no effect when targeted to other areas (including the head; higher doses localized to the head, pelvis, or thorax did produce aversions, but even here, they did not approximate those targeting the abdomen). Garcia and Kimeldorf noted that abdomen radiation induced aversions at the same dose that decreased gastric distension and transit, leading them to conclude that gastric disruption is the stimulus likely necessary to condition aversions. As other agents (mostly toxins) were reported to induce aversions, it was assumed that these compounds also produced sickness or malaise, but these conclusions were generally made in the absence of direct corroborative evidence of such effects (and often in the context of contrary evidence, e.g., the general inability of antiemetic drugs to affect CTAs [198, 199], the ability of antiemetics to induce CTAs themselves [109, 200], and the absence of a relationship between the degree of sickness and CTAs [201].

A similar explanation was used by many to account for the avoidance of taste paired with drugs of abuse which, in part, created the initial paradox of how drugs of abuse could be both aversive (via sickness) and rewarding at the same time. While conditioned aversions (sickness) were often applied to the suppression of consumption of tastes paired with drugs of abuse, others challenged this position. For example, in an elegant series of studies, Parker assessed taste

reactivity as an index of the sickness-inducing effects of drugs and found that although most drugs of abuse resulted in the suppression of consumption of solutions paired with the drug (see [202]; see also above; Table 2), these same drugs did not produce signs of sickness in the taste reactivity assessments ([203]; for recent work with LiCl vs. lactose, see [204]). In assessments of taste reactivity, a taste previously associated with some drug, e.g., LiCl, amphetamine, and cocaine, is infused into the animal's mouth via an indwelling cannula and both aversive and positive taste reactions to the infused solution are recorded. If the taste has been paired with LiCl (and other emetics), a host of aversive taste reactions are increased, e.g., gaping, chin wipes, and paw treading, which are used as an index of sickness/malaise induced by the conditioned taste (via its pairing with a drug that induced such effects unconditionally; see [205, 206]). As noted, tastes paired with drugs of abuse generally do not induce aversive taste reactivity (for reviews, see [207–209]). Parker and her colleagues concluded from these analyses that drugs of abuse do not induce sickness (as indexed by the taste reactivity test), and thus, the suppression of consumption of the taste associated with such drugs is not a function of a conditioned aversion to the taste itself. Importantly, Parker has shown that even drugs such as LiCl which do induce aversive taste reactivity (reflective of conditioned sickness) do not suppress the intake of LiCl-associated solutions through this mechanism in that antiemetics can attenuate aversive taste reactivity while leaving LiCl-taste aversions intact [210] (for a review, see [199]). From her analysis of the basis of the suppressed consumption of solutions induced by drugs of abuse, Parker suggests that sickness plays no role and even questions the role of sickness in suppression induced by emetics such as LiCl (for evidence critical of Parker's position of the importance of sickness in taste aversion learning using measures other than taste reactivity, i.e., lick pattern and rate, to assess sickness and palatability shifts, see [211–215]; see also [216, 217]).

Given the diminishing role of sickness as the common mediator of the effects induced by various agents to induce taste aversions, others turned to different mediators of such effects. In this context, it was generally stated that aversion-inducing agents had toxic (adverse) effects (but not necessarily sickness or malaise) that were responsible for taste aversion conditioning [31]. As such, interpretations became couched not in sickness but in rather general terms of toxicity. Such a general term conveyed no clear mechanisms of the toxicity, and, further, drugs with reported toxicity in other behavioral toxicological screens did not always induce aversions [41] (for a discussion, see [31]). Also, when drugs of abuse were reported to induce taste aversions (see above), the explanations were even more difficult in the context of general toxicity as such drugs in addition to being rewarding in other preparations produced no obvious toxic effects at the doses tested [128] (see [20]). To address this issue, a number of individuals noted that such drugs of abuse were aversive by disrupting normal homeostasis [31, 127, 128, 207, 214]. That is, given that general homeostasis is a well-defended state (see [128]; for related discussion on drugs of abuse in terms of their transition from use to abuse, see [5]); any disruption in this state is perceived as dangerous and defended. In one of the first reports on this possibility, Gamzu [128] discussed drug novelty itself as being the necessary condition for disruptions of homeostasis and in conditioning taste aversions (see [218] for a related position that argued that the actual rewarding effects of drugs were the novel stimulus that induced taste aversions). Although drug novelty is important in the conditioning of taste aversions (as exposure to the drug prior to conditioning weakens the acquisition of taste aversions), several arguments challenged this specific account. For example, as noted above, a number of classic toxins such as strychnine and cyanide as well as convulsants fail to induce CTAs (see [41]). It is difficult to explain how drugs with clear toxicity fail to induce a novel state. More importantly, although drug history weakens taste aversion acquisition, with repeated conditioning trials (where the taste and familiar drug are repeatedly paired), aversions do develop despite the fact that the drug is no longer novel (for a review, see [219]).

Disruptions in homeostasis can be produced by toxins, chemicals with adverse effects, and drugs of abuse, and according to the evolutionary importance of recognizing such disruptions as potentially dangerous, all such compounds should be effective in inducing taste aversions (and for the most part they are). However, stating that such disruptions are important in inducing aversions does not suggest that there is a common mechanism mediating all of these compounds. For example, even though drugs of abuse are rewarding as assessed in standard operant and Pavlovian designs that index these effects, collateral effects such as sickness (morphine), hyperthermia (synthetic cathinones [145]), anxiety (cocaine and amphetamine [138, 139]), sedation (barbiturates), hypothermia (alcohol [220]), and opponent process-related withdrawal (cocaine and morphine [136, 183, 221–223]), may be the stimuli important in inducing aversions by virtue of their ability to disrupt homeostasis. What is critical about this explanation of homeostasis is that the aversive effects of drugs that condition aversions are drug (and parameter)-specific (and not due to a general issue of malaise or stress; for a discussion of a potential common mediator, i.e., fear, see [224]). It is important to note here that few of the proposed mediating stimulus effects have been directly tested and the failure of specific toxins to induce aversions still needs to be explained (see [31] for an explanation of failure of several rodenticides and toxins). Further, over the past 20 years, a wide range of neuroactive compounds as well as neurochemicals involved in the modulation of normal neuronal function (and behaviorally active) are not effective in inducing CTAs, failures that challenge a simple homeostatic disruption as mediating aversion learning, e.g., interleukin-1B [225], interleukin-6 [226], leptin [227], GHR-R antagonist JMV 2959 [228, 229], L-tryptophan [230], N-acylphosphatidylethanolamine [231], and oleoylethanolamide [232].

Although each of these mechanistic accounts has been offered as a basis for taste aversion learning and most papers refer to one of these interpretations in their analysis of CTAs, no individual perspective is generally accepted.

Independent of which interpretation eventually garners consensus, all argue that the drug (whether a toxin, exogenous chemical agent, peptide, neurochemical, or drug of abuse) has some adverse effect that induces aversions (for an alternative interpretation that argues that the avoidance of drug-paired tastes is a function of a reward comparison in which the taste is devalued relative to the injected drug, i.e., anticipatory contrast, see [155–157, 233–235]; see [20] for a review of the reward comparison hypothesis).

12. Implications for the Aversive and Rewarding Effects of Drugs to Use and Abuse

The fact that drugs of abuse have both aversive and rewarding effects is now well characterized by a wide range of such compounds. The fact that the two effects are also dissociable is important given that they can be differentially impacted by a host of parametric, experiential, and subject factors (see above). That is, the balance between these two affective properties can be differentially impacted by these factors and, in turn, can change abuse vulnerability. In this context, the hypothetical interaction of aversion and reward as illustrated in Figure 3 is not static but is one that will differ depending upon the drug examined (including its dose, route of administration, and frequency of use) and the myriad of subject (sex, age, and genetics) and experiential (drug interactions, drug history, drug expectancy, and conditioning history) factors that can modulate each affective response (for a discussion, see [166, 176, 236]). Knowing the impact of these factors on drug aversion and reward and their balance should provide insight into the drug's use and its abuse vulnerability.

The questions then become how this impact occurs and under what conditions. The complexity of these questions becomes clear when one examines facets of drug-taking (see [4]) that range from controlled to dysregulated use (abuse). In this context, aversion and reward (and their balance) could impact the likelihood of initial drug intake and its maintenance as well as the dysregulation of drug intake that may escalate to abuse. Although each of these is important in assessing how drug use and abuse may be impacted by the affective properties of drugs, relatively little has addressed these specific issues. This review will highlight some of the work in these areas and what could be done.

These issues have recently been addressed by De Wit and Phillips [13] in their review "Do initial responses to drugs predict future drug use?." In this review, they raise the point that drugs can vary on a number of characteristics, including the magnitude, quality, and duration of their effects, all of which may impact subsequent use. One characteristic which is highlighted in their review is the affective valence of a drug's effects, i.e., the positive and negative effects that may facilitate and discourage use, respectively (similar to the affective properties noted in the present review). Using both retrospective and prospective assessments (along with human laboratory studies), they then assess for a variety of drugs (alcohol, nicotine, caffeine, psychostimulants, heroin, and marijuana) whether the initial response to these compounds is associated with subsequent use and/or abuse. As they describe, individuals differ significantly in their initial response to these drugs (as a function of environmental and genetic influences) and that for several drugs the initial responses (either positive or negative) are correlated with subsequent use (and in some instances substance use disorder). For example, with alcohol, individuals who initially display greater stimulant-like effects as breath alcohol concentrations are rising (see [237]) and feel fewer depressant effects as these levels decrease [238] are more likely to use and abuse alcohol. Conversely, those individuals that experience unpleasant effects are less likely to subsequently consume alcohol, effects and outcomes similar to what was previously described for individuals with metabolic differences in the ability to metabolize the alcohol metabolite acetaldehyde (see above; see also [22, 239]).

For other drugs (e.g., nicotine), the initial positive response was a better predictor of use and abuse (although negative responses limited intake under some conditions). For others (e.g., marijuana), only initial positive responses were associated with later use that, in turn, had little association with initial negative reactivity. For caffeine, unpleasant effects or negative subjective responses predicted lower consumption. Finally, for heroin, individuals having greater positive experience were more associated with later abuse (although no data have been reported on the relative association with any negative effects, e.g., nausea). In a summary of their work, De Wit and Phillips [13] cautioned that an understanding of the contribution of the initial affective response to a drug to its later use and/or abuse must be assessed in the context of many other factors such as expectancies, cognitive control, drug history, learning, and physical dependence, all of which clearly impact the likelihood of continued drug use and its escalation (e.g., the fact that individuals adjust doses of heroin or become tolerant to its aversive effects may limit generalization about the relative role of initial positive and negative effects to its continued use).

Interestingly, De Wit and Phillips [13] also assessed related work on positive and negative drug effects in animal models and noted the relative paucity of data assessing affective valence in nonhuman subjects and its relationship to drug intake in animals. The one area for which considerable data have been reported has used selectively bred animals (lines selectively bred for specific phenotypes) and inbred animal strains (derived from full-sibling mating that maximize genetic homogeneity) as their focus (for reviews, see [176, 236, 240]). The creation of selectively bred lines that are differentially sensitive to the aversive effects of drugs has been reported for many years (see [241, 242]). Such animals (taste aversion prone and taste aversion resistant, TAP and TAR, respectively) display significant differences in their ability to acquire aversions induced by a variety of drugs, e.g., cyclophosphamide, LiCl, and emetine hydrochloride [241, 242], and importantly by drugs of abuse, e.g., alcohol ([243]; see also [244]), with the TAP animals displaying aversions at lower doses and acquiring aversions at a faster rate than the TAR animals. These differences in aversion learning do not reflect differential abilities to learn in general

as TAP and TAR rats are similar in other learning preparations that do not utilize aversion conditioning (see [245, 246]).

Although Elkins and his group did not assess the potential contribution of these differential sensitivities of the aversive effects of alcohol (and cocaine) to drug intake in these same animals, others have done related work and have shown in both inbred strains and other selected lines that animals that show greater drug-induced taste aversions induced by specific compounds such as alcohol, methamphetamine, and heroin are less likely to self-administer those same compounds (orally or intravenously) (see [166, 176, 236]). For example, the Wistar Kyoto (WKY) rat strain that generally displays low consumption of alcohol in free-choice assessments displays strong taste aversions to novel solutions paired with exogenously administered ethanol (and differ significantly in both measures relative to the Marshal strain (M520) that generally consumes high levels of alcohol and only weak aversions to ethanol-paired solutions). In other words, there is an inverse relationship between alcohol consumption and its aversive effects (see [247, 248] for similar comparisons between the high alcohol-consuming Wistar Kyoto hyperactive rat (WKHA) and the WKY and spontaneously hypertensive rat strains).

The vast majority of work assessing the relationship between the aversive effects of drugs and drug intake has been with inbred strains of mice, specifically the C57BL/6J and DBA/2J strains. These two strains have been examined for alcohol preference and ethanol-induced taste aversions in a number of contexts and have consistently been shown to display an inverse relationship between alcohol intake and ethanol-induced taste aversions. Specifically, DBA mice (alcohol avoiding) acquire ethanol-induced taste aversions at a lower dose/concentration and at a faster rate relative to alcohol-preferring C57 mice. In a comprehensive analysis of 15 inbred mouse strains, Broadbent et al. [249] reported a significant inverse relationship between alcohol consumption and ethanol-induced taste aversions, suggesting that the sensitivity to the aversive effects of alcohol may serve as a protection against elevated alcohol intake (see [250] for a similar inverse relationship between oral alcohol intake and ethanol-induced place aversion conditioning, another index of the aversive effects of drugs; see [251] for related work showing that ethanol-induced place preference conditioning was not significantly correlated with alcohol consumption, suggesting a greater role for the aversive effects of alcohol in strain differences in alcohol acceptability).

Other work has focused on selective breeding to assess the relationships between the aversive effects of drugs and their self-administration. For example, Phillips and her colleagues have reported similar findings with methamphetamine. Specifically, rats that are selectively bred for high oral self-administration of methamphetamine were less likely to display methamphetamine-taste aversion (an index of its aversive effects) and more likely to display methamphetamine-place preferences (an index of its rewarding effects) than rats selectively bred for low methamphetamine intake (see [183]; see also [252, 253]). Interestingly, rats selectively bred for low and high drinking of methamphetamine displayed no differential aversive (as measured by CTA) or rewarding (as measured by CPP) effects of cocaine, showing that the genetic sensitivity to the aversive and rewarding effects of methamphetamine does not impact cocaine susceptibility [195]. Such findings of the inverse relationship between the aversive effects of a drug and its tendency to be self-administered in selectively bred strains are not limited to methamphetamine and the low and high drinking mice. For example, our laboratory has also focused on this issue in selectively bred rat strains, specifically the Lewis (LEW) and Fischer (F344) strains that are well characterized for their differences in intravenous self-administration of a variety of drugs. These two strains were originally selectively bred for cancer susceptibility and tissue inflammation (for discussion of the origins of these lines, see [176]) but subsequently were shown to differ for a myriad of other behaviors, including stress reactivity and drug intake, although the two strains were not selectively bred for differences in these latter two effects. In relation to drug intake, the LEW and F344 rat strains are well characterized for their differences in the self-administration of a variety of drugs of abuse, including alcohol (oral), morphine, etonitazene, methamphetamine, cocaine, and nicotine, and under most comparisons, LEW rats self-administer greater amounts of drugs than does the F344 strain (for a review, see [176]). The general conclusion regarding these genetic differences in drug intake is that the two strains differ significantly in their sensitivity to the drugs' rewarding effects, a conclusion supported by the fact that for a variety of drugs the LEW strain displays conditioned place preferences at lower doses than the F344 strain (see [176]). Interestingly, however, these strains also differ significantly in the aversive effects of the same drugs. For example, we have demonstrated for morphine [254–256], alcohol [163], and nicotine [257] that the F344 strain acquires morphine-, alcohol-, and nicotine-induced taste aversions at lower doses than the LEW strain. Such differences in sensitivity to the aversive effects of these drugs are not a general function of learning as these strains differ in the opposite direction for the drugs' rewarding effects as indexed by place preference conditioning (L > F). Further, the two strains do not differ in aversions induced by compounds with no abuse potential, e.g., the emetic LiCl [258], the kappa opiate receptor agonist U50,488H [255], the delta opiate receptor agonist SNC80 [255], and the peripherally acting mu opiate receptor agonist loperamide [256].

Thus, similar to work with inbred strains, we see an inverse relationship between drug intake and the relative sensitivity to taste aversion conditioning, i.e., animals readily self-administering the drug display weaker taste aversions (and vice versa), again substantiating a genetic component mediating the basis for drug use. In that context, there are several important caveats. First, when cocaine is used as the aversion-inducing agent, the LEW strain displays greater aversions, i.e., the LEW strain self-administers cocaine at higher rates than the F344 strain and displays greater cocaine-induced taste aversions [259] (see [260] for related findings with caffeine). While this challenges the inverse relationship reported with alcohol, morphine, and nicotine,

it should be noted that such a finding is not necessarily inconsistent with the position that drug intake is a balance of reward and aversion. In the case of cocaine, the LEW strain is more sensitive to both affective properties, but the balance may nonetheless be shifted toward reward, supporting greater self-administration (see above discussion on the balance of reward and aversion). A second caveat concerns the implications of the findings in general for a genetic mediation of the aversive and rewarding effects of drugs based on the selective breeding model. While the difference between the LEW and F344 rat strains clearly represents effects that have genetic influence, it does not mean that these differences cannot be impacted by environmental factors. Support for this position comes from work on cross-fostering in the strains. For example, we have reported that while LEW and F344 stains differ in their sensitivity to the aversive effects of morphine (F > L; see [254]), these differential sensitivities are partially reversed with cross-fostering. That is, F344 animals that normally display robust morphine-induced aversions resemble pups for the LEW strain that are relatively insensitive to morphine if they are reared by a LEW dam; conversely, LEW animals that generally show weak morphine-induced aversions acquire strong aversions (similar to the F344 strain) if reared by a F344 dam [261]. Partial reversals were also seen when cocaine was used as the aversion-inducing drug, i.e., the F344 strain that normally displays weak cocaine-induced taste aversion display strong aversion characteristics of the LEW strain if reared by a LEW dam (see [176]; for reports of reversals of other behavioral effects by cross-fostering, see [262, 263]).

The major work showing how the aversive (and rewarding) effects of drugs may impact drug intake has primarily been within various genetic models (see above; for related work with KO mice, see also [186, 189, 264–266]; see also [267, 268] for effects of aldehyde dehydrogenase type 2 KO and alcohol consumption and acetaldehyde brain, blood, and liver levels). It is important to note here that the aversive effects of drugs are impacted by a variety of other factors such as sex [177], drug history [219], and age [269, 270], and the impact of these factors on drug use and abuse has only recently been explored in this context. It will be critical in such analyses that the impact of their effects be explored not only on the initial use of various drugs of abuse (i.e., reflecting the drug's initial acceptability) but also on how these factors impact the likelihood of drug escalation and choice as well as relapse of extinguished drug response (reinstatement) (for an example of such assessments on the complexity of drug-taking, see [271]).

13. Drug Regulation

Although the aversive effects of a drug on its subsequent intake have primarily been discussed as a limiting or protecting factor (see [20, 141, 236]), these effects may also be important to the regulation of normal intake or dysregulation of intake that transitions drug use to drug abuse. This possibility has recently been suggested by our laboratory in discussions on the role of drug states in regulated drug intake [272, 273]. A principal feature of drug addiction is overconsumption followed by a reduced ability to control the desire to obtain drugs regardless of the risks involved, ultimately resulting in compulsive drug seeking [7]. The possibility that environmental stimuli associated with the postingestive rewarding effects of drugs strongly motivate consumption has guided much of the research exploring the role of neuroadaptations in the mesolimbic and mesocortical dopamine systems as well as in the prefrontal and orbitofrontal cortices mediating responses to drug rewards [5, 274]. There is little doubt that the memory of highly rewarding postingestive drug effects can strongly influence expectations about the outcomes a particular drug will produce. The strength to which drug-related contexts (e.g., time and space) can excite retrieval of those memories is a key determinant of current and future consummatory behavior [275, 276].

However, the response to drug-related cues involves more than excitatory associations that predict rewarding postingestive outcomes that, in turn, generate drug-taking. As demonstrated by the majority of individuals consuming drugs, the patterns of drug intake are generally well regulated, indicating that the capacity for drug-related cues to evoke intake is not without limits [277, 278]. That is, bouts of drug intake stop even when environmental cues (and the drug itself) that have gained the power to initiate intake are still present. Such regulatory control involves higher-level learning and memory processes that counter the power of palatable drugs (and drug-related stimuli) to inhibit further intake [271, 279–281]. The same environmental stimuli associated with the rewarding consequences on some occasions, e.g., at the outset of consumption, can also predict nonrewarding or even aversive consequences on other occasions, e.g., toward the end of a bout of drug use. Thus, both excitatory and inhibitory associations are formed between drug cues and positive and negative postingestive outcomes, respectively, depending on the context. The ambiguous nature of the associations between drug-related cues and these consequences suggests that additional signals must be used to predict when taking the drug will produce rewarding outcomes and when the drug will produce nonrewarding or even aversive effects, leading to an end in the bout of drug intake. We are suggesting that regulatory control of drug intake is dependent in part on the ability of contextual drug states to disambiguate conflicting associations between drug cues and postingestion outcomes. In this framework, although the initiation of drug-taking depends on the activation of excitatory associations that predict rewarding effects, contextual stimuli suppress intake by activating the inhibitory associations between the same drug cues associated with nonrewarding or aversive postingestive outcomes. Dysregulated intake, in turn, may be a function of poor control by these drug state cues that would produce an imbalance that favors the excitatory associations, leading to overconsumption (see evidence by [282–284] for the role of similar processes involved in the regulation/dysregulation of food intake).

Although the processes of cognitive inhibition described above have primarily been implicated in the regulation of food intake, the imbalance between inhibitory and excitatory

control mechanisms may also explain why some people progress from regulated to dysregulated drug use. Given free drug access, animals learn to regulate drug intake to achieve a particular level of intoxication by titrating drug levels within the brain and bloodstream [15]. Characteristics of maintained drug self-administration indicate that drug or drug-associated stimuli encountered by an animal in a drug-free state will initiate drug intake, but when the drug level reaches above a satiety threshold, i.e., the minimal drug level at which self-administration is maintained, animals temporarily suspend drug seeking [15, 280]. Consistent with this position, the presence or absence of a drug in the biological system has been demonstrated to serve discriminative functions that signal the availability of certain reinforcers, e.g., food and water [285–288]. Not only can drugs serve as discriminative stimuli in general but also the discriminative control of the training drug can also be generalized to drugs with comparable mechanisms of action [289–292].

We [293] and others [294–297] have extended the analysis on drug discrimination learning to show that interoceptive drug cues can also signal the presence and absence of an aversive outcome using a modified conditioned taste aversion design (for a review of the conditioned taste aversion baseline of drug discrimination learning, see [272]). In one demonstration, rats received PCP followed by a pairing of saccharin with the emetic LiCl on the conditioning day, and on the subsequent 3 days, the same animals received the PCP vehicle followed by paring of saccharin with the LiCl vehicle. Over multiple trials, animals avoided consuming saccharin when it was preceded by PCP and consumed saccharin when it was preceded by the PCP vehicle. The control group that received the same PCP/vehicle injections prior to saccharin, but never the postsaccharin injection of LiCl, consumed high levels of saccharin throughout the study, indicating that the suppressed saccharin consumption in the LiCl-treated group was a function of PCP signalling the saccharin-LiCl contingency rather than an unconditioned suppression on saccharin consumption [293] (for related work with morphine, see [295, 297]).

To date, accumulating evidence that a wide range of drugs can serve such a discriminative function provides clear support for the ability of drug stimuli to modulate consummatory behavior by signalling postingestive outcomes. Similar to the function of satiety cues in food intake regulation, discriminative learning may be important in regulating drug intake. Humans and other animals might use interoceptive drug signals to determine when a sufficient drug level has been achieved and whether to continue or refrain from continued or elevated consumption (see [13] for a discussion of the possible role of the inability to detect such stimulus effects in alcoholics). Such an evaluation of drugs and consequences of intake is governed by prior learning of various consequences of drug use. Further, memory processes represent associations between drug-taking contexts, drug cues, and the consequences of drug-taking acquired over a repeated experience that strongly influence the cognitive inhibition of drug intake. A rich literature has demonstrated that the hippocampus modulates the capacity of any contextual or discrete cues to activate inhibitory associations to suppress the retrieval of memories of rewarding outcomes [298–300]. Hippocampal damage has been shown to impair the ability of humans [301–303] and rats [304, 305] to discriminate interoceptive satiety cues from hunger and to suppress food-reinforced conditioned response overconsumption. Rats with impaired hippocampal-dependent inhibitory control showed greater intake and meal frequency within a shorter interval [306–308] and greater weight gain within weeks [309] or months [310].

Specific to the issue of drug intake, we suggest that neuroadaptations resulting from chronic drug use disrupt the ability of the hippocampus to mediate inhibitory control over responses to drug-related cues. In this context, hippocampal impairments have been implicated to modulate the transition from regulated to dysregulated drug intake. In chronic drug users, various drugs of abuse have been reported to impair the functional integrity of the hippocampus and interfere with learning and memory [311, 312]. Hippocampal lesions have also been demonstrated to increase the oral consumption of alcohol and the intravenous self-administration of cocaine, methamphetamine, morphine, and nicotine in rodents [313–316]. Furthermore, we have recently reported that chronic administration of cocaine interfered with the ability of rats to solve a hippocampal-dependent discriminative task known as the serial feature negative (sFN) discrimination that requires the animals to use contextual signals to disambiguate postingestive outcomes [317]. Interestingly, chronic administration of cocaine in the same animals had no effect on simple discrimination problem that does not require a functional hippocampus, indicating that the type of learning and memory processes required to resolve approach-avoidance conflict in the sFN discrimination task does not rely on nonspecific factors (e.g., motivation and attention) but a higher-level regulatory mechanism dependent on the hippocampus to disambiguate conflicting associations.

Taken together, these data highlight the fact that the hippocampus is critically involved in regulating consummatory behaviors and that chronic drug use uniquely targets hippocampal-dependent forms of learning and memories. If chronic drug use disrupts the hippocampal function to modulate the ability of interoceptive cues of drug satiety to engage inhibitory associations, then chronic drug exposure might reduce the power of drug satiety cues to inhibit intake. In support of this idea, future studies in drug addiction should not be limited to reward- and motivation-based models; rather, future research should also explore a model predicated upon hippocampal functioning underlying regulated drug use that could potentially contribute to preventing the transition to dysregulated drug use and pathological state of drug abuse.

14. Conclusions

In this review, we have discussed drug use and abuse in the context of a reward model and have qualified this analysis by arguing that drugs of abuse have multiple stimulus properties (both rewarding and aversive) that need to be considered in any account of drug-taking behavior. We provided

evidence that drugs have aversive effects (from both clinical and preclinical populations) and have introduced and discussed these effects in their historical context (generated by work on conditioned taste aversions). We indicate that the aversive effects can occur concurrent with rewarding ones in the same subject trained and tested under identical conditions and that their relative balance is important to the use and/or abuse of the drug. While both rewarding and aversive effects occur, we describe reports indicating that these effects appear dissociable, suggesting that manipulations can impact them differently. We further suggest that an awareness of each affective property and the multiple parametric, experiential, and subject factors that impact them and their relative balance will give insight into use and abuse vulnerability (for example, see [177]). Data supporting such a position were provided by an overview of the relationship between initial and subsequent drug use (in humans) and a more detailed analysis of the inverse relationship between perceived aversive effects of drugs and their intake. We close our review by noting that the interaction of reward and aversion may also be involved within bouts of drug intake as the ability to use the drug state itself to set the occasion for aversive effects that may accompany elevated use. We conclude from these issues that examining a drug's aversive effects (in addition to the myriad of other stimulus effects produced) is critical to understanding drug intake and developing prevention and treatment strategies associated with the transition from use to abuse.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References

[1] L. D. Johnston, R. A. Miech, P. M. O'Malley, J. G. Bachman, J. E. Schulenberg, and M. E. Patrick, *Monitoring the Future National Survey Results on Drug Use, 1975-2020*, Institute for Social Research, The University of Michigan, Michigan, USA, 2021.

[2] Substance Abuse and Mental Health Services Administration [SAMHSA], *Key substance use and mental health indicators in the United States: results from the 2020 National Survey on Drug Use and Health*, Center for Behavioral Health Statistics and Quality, Substance Abuse and Mental Health Services Administration, Rockville, MD, 2021.

[3] United Nations Office on Drugs and Crime (UNODC), *World Drug Report 2021*, United Nations Publication, 2021, Sales No. E.21.XI.8.

[4] N. D. Volkow, G. F. Koob, and A. T. McLellan, "Neurobiologic advances from the brain disease model of addiction," *New England Journal of Medicine*, vol. 374, pp. 363–371, 2016.

[5] G. F. Koob, M. A. Arends, and M. Le Moal, *Drugs, Addiction and the Brain*, Academic Press, Waltham, MA, 2014.

[6] G. F. Koob, "The dark side of emotion: the addiction perspective," *European Journal of Pharmacology*, vol. 753, pp. 73–87, 2015.

[7] G. F. Koob and N. D. Volkow, "Neurobiology of addiction: a neurocircuitry analysis," *The Lancet Psychiatry*, vol. 3, pp. 760–773, 2016.

[8] J. S. Meyer and L. F. Quenzer, *Psychopharmacology: Drugs, the Brain, and Behavior*, Sinauer Associates, Inc, Sunderland, MA, 2nd edition, 2018.

[9] E. C. O'Connor, K. Chapman, P. Butler, and A. N. Mead, "The predictive validity of the rat self-administration model for abuse liability," *Neuroscience & Biobehavioral Reviews*, vol. 35, pp. 912–938, 2011.

[10] B. Kuhn, P. Kalivas, and A. C. Bobadilla, "Understanding addiction using animal models," *Frontiers in Behavioral Neuroscience*, vol. 13, pp. 1–24, 2019.

[11] M. A. Smith, "Nonhuman animal models of substance use disorders: translational value and utility to basic science," *Drug and Alcohol Dependence*, vol. 206, article 107733, 2020.

[12] Y. Swain, J. C. Gewirtz, and A. C. Harris, "Behavioral predictors of individual differences in opioid addiction vulnerability as measures using i.v. self-administration in rats," *Drug and Alcohol Dependence*, vol. 221, article 108561, 2021.

[13] H. De Wit and T. J. Phillips, "Do initial responses to drugs predict future use or abuse?," *Neuroscience & Biobehavioral Reviews*, vol. 36, pp. 1565–1576, 2012.

[14] A. Goldstein, *Addiction: From Biology to Drug Policy*, Oxford University Press, Inc, New York, NY, 2nd edition, 2001.

[15] W. J. Lynch and M. E. Carroll, "Regulation of drug intake," *Experimental and Clinical Psychopharmacology*, vol. 9, pp. 131–143, 2001.

[16] J. R. DiFranza, J. A. Savageau, K. Fletcher et al., "Susceptibility to nicotine dependence: the development and assessment of nicotine dependence in youth 2 study," *Pediatrics*, vol. 120, pp. e974–e983, 2007.

[17] J. R. DiFranza, J. A. Savageau, K. Fletcher, J. K. Ockene, and N. A. Rigott, "Recollections and repercussions of the first inhaled cigarette," *Addictive Behaviors*, vol. 29, no. 2, pp. 261–272, 2004.

[18] J. R. DiFranza, J. A. Savageau, N. A. Rigotti et al., "Trait anxiety and nicotine dependence in adolescents: a report from the DANDY study," *Addictive Behaviors*, vol. 29, pp. 911–919, 2004.

[19] L. Lasagna, J. M. von Felsinger, and H. K. Beecher, "Drug-induced mood changes in man. I. Observations on healthy subjects, chronically ill patients, and postaddicts," *Journal of the American Medical Association*, vol. 157, pp. 1006–1020, 1955.

[20] A. Verendeev and A. L. Riley, "Conditioned taste aversion and drugs of abuse: history and interpretation," *Neuroscience & Biobehavioral Reviews*, vol. 36, pp. 2193–2205, 2012.

[21] S. J. Kohut and A. L. Riley, "Conditioned taste aversions and the assessment of the aversive effects of drugs: implications for drug use and abuse," in *Encyclopedia of Psychopharmacology*, I. P. Stolerman, Ed., Springer Verlag, Berlin, Germany, 2010.

[22] R. F. Suddendorf, "Research on alcohol metabolism among Asians and its implications for understanding causes of alcoholism," *Public Health Reports*, vol. 104, pp. 615–620, 1989.

[23] C. Cannizzaro, F. Plescia, and S. Cacace, "Role of acetaldehyde in alcohol addiction: current evidence and future perspectives," *Malta Medical Journal*, vol. 23, pp. 27–31, 2011.

[24] T. B. Baker and D. S. Cannon, "Alcohol and taste-mediated learning," *Addictive Behaviors*, vol. 7, pp. 211–230, 1982.

[25] A. W. Logue, K. R. Logue, and K. E. Strauss, "The acquisition of taste aversions in humans with eating and drinking disorders," *Behaviour Research and Therapy*, vol. 21, pp. 275–289, 1983.

[26] F. Lemere and W. L. Voegtlin, "Conditioned reflex therapy of alcoholic addiction: specificity of conditioning against chronic alcoholism," *California and Western Medicine*, vol. 53, pp. 268-269, 1940.

[27] W. L. Voegtlin, "The treatment of alcoholism by establishing a conditioned reflex," *American Journal of the Medical Sciences*, vol. 199, pp. 802–809, 1940.

[28] W. L. Voegtlin, F. Lemere, W. R. Broz, and P. O'Hollaren, "Conditioned reflex therapy of chronic alcoholism; IV. A preliminary report on the value of reinforcement," *Quarterly Journal of Studies on Alcohol*, vol. 2, pp. 505–511, 1941.

[29] R. L. Elkins, "An appraisal of chemical aversion (emetic therapy) approaches to alcoholism treatment," *Behaviour Research and Therapy*, vol. 29, pp. 387–413, 1991.

[30] S. H. Revusky, "Chemical aversion treatment of alcoholism," in *Conditioned Taste Aversion: Behavioral and Neural Processes*, S. Reilly and T. R. Schactman, Eds., pp. 445–472, Oxford University Press, New York, NY, 2009.

[31] A. L. Riley and D. L. Tuck, "Conditioned taste aversions: a behavioral index of toxicity," *Annals of the New York Academy of Sciences*, vol. 5, pp. 272–292, 1985.

[32] J. Rzóska, "Bait shyness, a study in rat behavior," *The British Journal of Animal Behaviour*, vol. 1, pp. 128–135, 1954.

[33] J. Garcia, D. J. Kimeldorf, and R. A. Koelling, "Conditioned aversion to saccharin resulting from exposure to gamma radiation," *Science*, vol. 122, pp. 157-158, 1955.

[34] K. B. Freeman and A. Riley, "The origins of conditioned taste aversion learning: a historical analysis," in *Conditioned Taste Aversion: Behavioral and Neural Processes*, S. Reilly and T. R. Schactman, Eds., pp. 9–33, Oxford University Press, New York, NY, 2009.

[35] J. Rzóska, "Stomach analysis of brown rats poisoned in the field," in *Control of Rats and Mice*, D. Chitty, Ed., pp. 395–413, Clarendon Press, Oxford, England, 1954.

[36] J. Garcia, F. R. Ervin, and R. A. Koelling, "Learning with prolonged delay of reinforcement," *Psychonomic Science*, vol. 5, pp. 121-122, 1966.

[37] J. Garcia and R. A. Koelling, "Relation of cue to consequence in avoidance learning," *Psychonomic Science*, vol. 4, pp. 123-124, 1966.

[38] J. Garcia and F. R. Ervin, "Gustatory-visceral and telereceptor-cutaneous conditioning—adaptation in internal and external milieus," *Communications in Behavioral Biology*, vol. 1, pp. 389–415, 1968.

[39] S. Revusky and J. Garcia, "Learned associations over long delays," in *Psychology of Learning and Motivation: Advances in Research and Theory*, G. H. Bower and J. T. Spence, Eds., pp. 1–84, Academic Press, Cambridge, MA, 1970.

[40] P. Rozin and J. W. Kalat, "Specific hungers and poison avoidance as adaptive specializations of learning," *Psychological Review*, vol. 78, pp. 459–486, 1971.

[41] M. Nachman and P. L. Hartley, "Role of illness in producing learned taste aversions in rats: a comparison of several rodenticides," *Journal of Comparative and Physiological Psychology*, vol. 89, pp. 1010–1018, 1975.

[42] R. C. MacPhail, "Studies on the flavor aversions induced by trialkyltin compounds," *Neurobehavioral Toxicology & Teratology*, vol. 4, pp. 225–230, 1982.

[43] A. L. Riley, R. J. Dacanay, and J. P. Mastropaolo, "The effects of trimethyltin chloride on the acquisition of long delay conditioned taste aversion learning in the rats," *Neurotoxicology*, vol. 5, pp. 291–295, 1984.

[44] M. J. Kallman, M. R. Lynch, and M. R. Landauer, "Taste aversions to several halogenated hydrocarbons," *Neurobehavioral Toxicology and Teratology*, vol. 5, pp. 23–27, 1983.

[45] L. K. Nicolaus, "Conditioned aversions in a guild of egg predators: implications for aposematism and prey defense mimicry," *American Midland Naturalist*, vol. 117, pp. 405–419, 1987.

[46] P. O. Sjödén and T. Archer, "Conditioned taste aversion to saccharin induced by 2,4,5-trichlorophenoxyacetic acid in albino rats," *Physiology & Behavior*, vol. 19, no. 1, pp. 159–161, 1977.

[47] S. Mahiout and R. Pohjanvirta, "Aryl hydrocarbon receptor agonists trigger avoidance of novel food in rats," *Physiology & Behavior*, vol. 167, pp. 49–59, 2016.

[48] Z. W. Brown, Z. Amit, B. Smith, and G. E. Rockman, "Differential effects on conditioned taste aversion learning with peripherally and centrally administered acetaldehyde," *Neuropharmacology*, vol. 17, pp. 931–935, 1978.

[49] A. Ungerer, D. Marchi, P. Ropartz, and J.-H. Weil, "Aversive effects and retention impairment induced by acetoxycycloheximide in an instrumental task," *Physiology & Behavior*, vol. 15, pp. 55–62, 1975.

[50] C. E. Anderson, H. A. Tilson, and C. L. Mitchell, "Conditioned taste aversion following acutely administered acrylamide," *Neurobehavioral Toxicology & Teratology*, vol. 4, pp. 497–499, 1982.

[51] I. L. Bernstein, M. V. Vitiello, and R. A. Sigmundi, "Effects of tumor growth on taste-aversion learning produced by antitumor drugs in the rat," *Physiological Psychology*, vol. 8, pp. 51–55, 1980.

[52] V. A. Rappold, J. H. Porter, and G. C. Llewellyn, "Evaluation of the toxic effects of aflatoxin B_1 with a taste aversion paradigm in rats," *Neurobehavioral Toxicology and Teratology*, vol. 6, pp. 51–58, 1984.

[53] K. H. Brookshire, C. N. Stewart, and H. N. Bhagavan, "Saccharin aversion in alloxan-diabetic rats," *Journal of Comparative and Physiological Psychology*, vol. 79, pp. 385–393, 1972.

[54] M. G. Hotchkiss, D. S. Best, R. L. Cooper, and S. C. Laws, "Atrazine does not induce pica behavior at doses that increase hypothalamic–pituitary–adrenal axis activation and cause conditioned taste avoidance," *Neurotoxicology and Teratology*, vol. 34, pp. 295–302, 2012.

[55] W. Ebeling, "The cockroach learns to avoid insecticides," *California Agriculture*, vol. 23, pp. 12–15, 1969.

[56] G. Ward-Fear, D. J. Pearson, G. P. Brown, B. Rangers, and R. Shine, "Ecological immunization: in situ training of free-ranging predatory lizards reduces their vulnerability to invasive toxic prey," *Biology Letters*, vol. 12, 2017.

[57] P. J. Wellman, P. A. Watkins, J. R. Nation, and D. E. Clark, "Conditioned taste aversion in the adult rat induced by dietary ingestion of cadmium or cobalt," *Neurotoxicology*, vol. 5, pp. 81–90, 1984.

[58] R. C. MacPhail and D. J. Leander, "Flavor aversion induced by chlordimeform," *Neurobehavioral Toxicology*, vol. 2, pp. 363–365, 1980.

[59] M. R. Landauer, T. W. Tomlinson, R. L. Balster, and R. C. MacPhail, "Some effects of the formamidine pesticide chlordimeform on the behavior of mice," *Neurotoxicology*, vol. 5, pp. 91–100, 1984.

[60] S. Revusky and S. Reilly, "Attenuation of conditioned taste aversions by external stressors," *Pharmacology Biochemistry and Behavior*, vol. 33, pp. 219–226, 1989.

[61] O. Buresová and J. Bures, "Conditioned taste aversion induced in rats by intracerebral or systemic administration of monoamine oxidase inhibitors," *Psychopharmacology*, vol. 91, pp. 209–212, 1987.

[62] S. Islam, "Snake neurotoxins and conditioned taste aversion in mice," *International Journal of Neuroscience*, vol. 11, pp. 41–43, 1980.

[63] C. E. O'Connor and L. R. Matthews, "Cyanide induced aversions in the possum (*Trichosurus vulpecula*): effect of route of administration, dose, and formulation," *Physiology & Behavior*, vol. 58, pp. 265–271, 1995.

[64] D. A. Booth and P. C. Simson, "Aversion to a cue acquired by its association with effects of an antibiotic in the rat," *Journal of Comparative and Physiological Psychology*, vol. 2, pp. 319–323, 1973.

[65] W. Dragoin, G. E. McCleary, and P. McCleary, "A comparison of two methods of measuring conditioned taste aversions," *Behavior Research Methods & Instrumentation*, vol. 3, pp. 309-310, 1971.

[66] S. Revusky and G. M. Martin, "Glucocorticoids attenuate taste aversions produced by toxins in rats," *Psychopharmacology*, vol. 96, pp. 400–407, 1988.

[67] A. El Hani, R. J. Mason, D. L. Nolte, and R. H. Schmidt, "Flavor avoidance learning and its implications in reducing strychnine baiting hazards to nontarget animals," *Physiology & Behavior*, vol. 64, pp. 585–589, 1998.

[68] D. S. Cannon and T. B. Baker, "Emetic and electric shock alcohol aversion therapy: assessment of conditioning," *Journal of Consulting and Clinical Psychology*, vol. 49, pp. 20–33, 1981.

[69] M. Irie, S. Asami, S. Nagata, M. Miyata, and H. Kasai, "Classical conditioning of oxidative DNA damage in rats," *Neuroscience Letters*, vol. 288, pp. 13–16, 2000.

[70] E. M. Stricker and N. E. Wilson, "Salt-seeking behavior in rats following acute sodium deficiency," *Journal of Comparative and Physiological Psychology*, vol. 72, pp. 416–420, 1970.

[71] J. A. Rudd, M. P. Ngan, and M. K. Wai, "5-HT_3 receptors are not involved in conditioned taste aversions induced by 5-hydroxytryptamine, ipecacuanha or cisplatin," *European Journal of Pharmacology*, vol. 352, pp. 143–149, 1998.

[72] J. D. Leander and B. A. Gau, "Flavor aversions rapidly produced by inorganic lead and triethyltin," *Neurotoxicology*, vol. 1, pp. 635–642, 1980.

[73] M. S. Exton, D. F. Bull, M. G. King, and A. J. Husband, "Paradoxical conditioning of the plasma copper and corticosterone responses to bacterial endotoxin," *Pharmacology Biochemistry and Behavior*, vol. 52, pp. 347–354, 1995.

[74] S. B. Klein, M. J. Barter, A. L. Murphy, and J. H. Richardson, "Aversion to low doses of mercuric chloride in rats," *Physiological Psychology*, vol. 2, pp. 397–400, 1974.

[75] M. Miyagawa, "Conditioned taste aversion induced by inhalation exposure to methyl bromide in rats," *Toxicology Letters*, vol. 10, no. 4, pp. 411–416, 1982.

[76] T. E. Levine, "Conditioned aversion following ingestion of methylmercury in rats and mice," *Behavioral Biology*, vol. 22, pp. 489–496, 1978.

[77] R. J. Mason and R. F. Reidinger, "Observational learning of food aversions in red-winged blackbirds (*Agelaius phoeniceus*)," *The Auk*, vol. 99, pp. 548–554, 1982.

[78] J. R. Millner and T. Palfai, "Metrazol impairs conditioned aversion produced by LiCl: a time dependent effect," *Pharmacology Biochemistry and Behavior*, vol. 3, pp. 201–204, 1975.

[79] C. R. Gustavson, G. A. Holzer, J. C. Gustavson, and D. L. Vakoch, "An evaluation of phenol methylcarbamates as taste aversion producing agents in caged blackbirds," *Applied Animal Ethology*, vol. 8, pp. 551–559, 1982.

[80] M. Nachman, D. Lester, and J. L. Magnen, "Alcohol aversion in the rat: behavioral assessment of noxious drug effects," *Science*, vol. 168, pp. 1244–1246, 1970.

[81] T. Archer, A. K. Mohammed, and T. U. C. Jarbe, "Latent inhibition following systemic DSP4: effects due to presence and absence of contextual cues in taste-aversion learning," *Behavioral and Neural Biology*, vol. 38, pp. 287–306, 1983.

[82] D. E. Clark and P. J. Wellman, "Conditioned saccharin taste aversion induced by mycotoxins in rats: lack of effect of ochratoxin A," *Pharmacology Biochemistry and Behavior*, vol. 32, pp. 819–821, 1989.

[83] R. C. MacPhail and D. B. Peele, "Animal models for assessing the neurobehavioral impact of airborne pollutants," *Annals of the New York Academy of Sciences*, vol. 30, pp. 294–303, 1992.

[84] M. S. Dey, R. I. Krieger, and R. C. Ritter, "Paraquat-induced, dose-dependent conditioned taste aversions and weight loss mediated by the area postrema," *Toxicology and Applied Pharmacology*, vol. 87, pp. 212–221, 1987.

[85] S. J. St. John, L. Pour, and J. D. Boughter, "Phenylthiocarbamide produces conditioned taste aversions in mice," *Chemical Senses*, vol. 30, pp. 377–382, 2005.

[86] J. A. Chester and C. L. Cunningham, "Baclofen alters ethanol-stimulated activity but not conditioned place preference or taste aversion in mice," *Pharmacology Biochemistry and Behavior*, vol. 63, pp. 325–331, 1999.

[87] M. R. Landauer and J. A. Romano, "Acute behavioral toxicity of the organophosphate sarin in rats," *Neurobehavioral Toxicology & Teratology*, vol. 6, pp. 239–243, 1984.

[88] J. A. Romano, J. M. King, and D. M. Penetar, "A comparison of physostigmine and soman using taste aversion and nociception," *Neurobehavioral Toxicology & Teratology*, vol. 7, pp. 243–249, 1985.

[89] A. W. Kusnecov, R. Liang, and G. Shurin, "T-lymphocyte activation increases hypothalamic and amygdaloid expression of CRH mRNA and emotional reactivity to novelty," *Journal of Neuroscience*, vol. 11, pp. 4533–4543, 1999.

[90] W. E. Howard, S. D. Palmateer, and M. Nachman, "Aversion to strychnine sulfate by Norway rats, roof rats, and pocket gophers," *Toxicology and Applied Pharmacology*, vol. 12, pp. 229–241, 1968.

[91] P. J. Wellman, L. D. Rowe, D. E. Clark, and R. D. Cockroft, "Effects of T-2 toxin on saccharin aversion and food consumption in adult rats," *Physiology & Behavior*, vol. 45, pp. 501–506, 1989.

[92] C. R. Gustavson, J. C. Gustavson, and G. A. Holzer, "Thiabendazole-based taste aversions in dingoes (*Canis familiaris dingo)* and new guinea wild dogs (*Canis familiaris*

hallstromi)," *Applied Animal Ethology*, vol. 10, pp. 385–388, 1983.

[93] J. Tobajas, P. Gómez-Ramírez, P. María-Mojica et al., "Selection of new chemicals to be used in conditioned aversion for non-lethal predation control," *Behavioural Processes*, vol. 166, article 103905, 2019.

[94] L. E. Goehler, C. R. Busch, N. Tartaglia et al., "Blockade of cytokine induced conditioned taste aversion by subdiaphragmatic vagotomy: further evidence for vagal mediation of immune-brain communication," *Neuroscience Letters*, vol. 185, no. 163, p. 166, 1995.

[95] R. L. Balster and J. F. Borzelleca, "Behavioral toxicity of trihalomethane contaminants of drinking water in mice," *Environmental Health Perspectives*, vol. 46, pp. 127–136, 1982.

[96] M. Miyagawa, T. Honma, M. Sato, and H. Hasegawa, "Conditioned taste aversion induced by toluene administration in rats," *Neurobehavioral Toxicology and Teratology*, vol. 6, pp. 33–37, 1984.

[97] D. E. Clark, P. J. Wellman, R. B. Harvey, and M. S. Lerma, "Effects of vomitoxin (deoxynivalenol) on conditioned saccharin aversion and food consumption in adult rats," *Pharmacology Biochemistry and Behavior*, vol. 27, pp. 247–252, 1987.

[98] S. E. Baker, S. A. Ellwood, R. W. Watkins, and D. W. Macdonald, "A dose-response trial with ziram-treated maize and free-ranging European badgers *Meles meles*," *Applied Animal Behaviour Science*, vol. 93, pp. 309–321, 2005.

[99] J. K. Gore-Langton, S. M. Flax, R. L. Pomfrey, B. B. Wetzell, and A. L. Riley, "Measures of the aversive effects of drugs: a comparison of conditioned taste and place aversions," *Pharmacology Biochemistry and Behavior*, vol. 134, pp. 99–105, 2015.

[100] D. Lester, M. Nachman, and J. Le Magnen, "Aversive conditioning by ethanol in the rat," *Quarterly Journal of Studies on Alcohol*, vol. 31, pp. 578–586, 1970.

[101] H. Cappell and A. E. LeBlanc, "Conditioned aversion to saccharin by single administrations of mescaline and d-amphetamine," *Psychopharmacologia*, vol. 22, pp. 352–356, 1971.

[102] H. Cappell, A. E. LeBlanc, and L. Endrenyi, "Aversive conditioning by psychoactive drugs: effects of morphine, alcohol and chlordiazepoxide," *Psychopharmacologia*, vol. 29, pp. 239–246, 1973.

[103] M. A. Bozarth, *Methods of Assessing the Reinforcing Properties of Abused Drugs*, Springer-Verlag, New York, NY, 1987.

[104] K. H. Nelson, B. J. Hempel, M. M. Clasen, K. C. Rice, and A. L. Riley, "Conditioned taste avoidance, conditioned place preference and hyperthermia induced by the second generation 'bath salt' α-pyrrolidinopentiophenone (α-PVP)," *Pharmacology Biochemistry and Behavior*, vol. 156, pp. 48–55, 2017.

[105] T. F. Elsmore and G. V. Fletcher, "Δ^9-Tetrahydrocannabinol: aversive effects in rat at high doses," *Science*, vol. 175, pp. 911-912, 1972.

[106] H. Q. Lin, D. M. Atrens, M. J. Christie, D. M. Jackson, and I. S. McGregor, "Comparison of conditioned taste aversions produced by MDMA and d-amphetamine," *Pharmacology Biochemistry and Behavior*, vol. 46, pp. 153–156, 1993.

[107] H. E. King, B. Wetzell, K. C. Rice, and A. L. Riley, "3, 4-Methylenedioxypyrovalerone (MDPV)-induced conditioned taste avoidance in the F344/N and LEW rat strains," *Pharmacology Biochemistry and Behavior*, vol. 126, pp. 163–169, 2014.

[108] J. R. Vogel and B. A. Nathan, "Learned taste aversions induced by hypnotic drugs," *Pharmacology Biochemistry and Behavior*, vol. 3, pp. 189–194, 1975.

[109] B. D. Berger, "Conditioning of food aversions by injections of psychoactive drugs," *Journal of Comparative and Physiological Psychology*, vol. 81, pp. 21–26, 1972.

[110] F. B. Jolicoeur, D. B. Rondeau, M. J. Wayner, R. B. Mintz, and A. D. Merkel, "Barbiturates and alcohol consumption," *Biobehavioral Reviews*, vol. 1, pp. 177–196, 1977.

[111] G. Dickens and W. H. Trethowan, "Cravings and aversions during pregnancy," *Journal of Psychosomatic Research*, vol. 15, pp. 259–268, 1971.

[112] A. J. Goudie and T. Newton, "The puzzle of drug-induced conditioned taste aversion: comparative studies with cathinone and amphetamine," *Psychopharmacology*, vol. 87, pp. 328–333, 1985.

[113] M. E. Corcoran, I. Bolotow, Z. Amit, and J. A. McCaughran Jr., "Conditioned taste aversions produced by active and inactive cannabinoids," *Pharmacology Biochemistry and Behavior*, vol. 2, pp. 725–728, 1974.

[114] A. J. Goudie, D. W. Dickins, and E. W. Thornton, "Cocaine-induced conditioned taste aversions in rats," *Pharmacology Biochemistry and Behavior*, vol. 8, pp. 757–761, 1978.

[115] I. S. McGregor, C. N. Issakidis, and G. Prior, "Aversive effects of the synthetic cannabinoid CP 55,940 in rats," *Pharmacology Biochemistry and Behavior*, vol. 53, pp. 657–664, 1996.

[116] G. D. Busse, A. Verendeev, J. Jones, and A. L. Riley, "The effects of cocaine, alcohol and cocaine/alcohol combinations in conditioned taste aversion learning," *Pharmacology Biochemistry and Behavior*, vol. 82, pp. 207–214, 2005.

[117] P. S. Grigson, R. C. Twining, and R. M. Carelli, "Heroin-induced suppression of saccharin intake in water-deprived and water-replete rats," *Pharmacology Biochemistry and Behavior*, vol. 66, pp. 603–608, 2000.

[118] A. L. Riley, K. H. Nelson, M. E. Crissman, and K. A. Pescatore, "Conditioned taste avoidance induced by the combination of heroin and cocaine: implications for the use of speedball," *Pharmacology Biochemistry and Behavior*, vol. 187, article 172801, 2019.

[119] F. Etscorn and P. Parson, "Taste aversion in mice using phencyclidine and ketamine as the aversive agents," *Bulletin of the Psychonomic Society*, vol. 14, pp. 19–21, 1979.

[120] R. J. Carey and E. B. Goodall, "Amphetamine-induced taste aversion: a comparison of d-versus l-amphetamine," *Pharmacology Biochemistry and Behavior*, vol. 2, pp. 325–330, 1974.

[121] L. A. Parker, "LSD produces place preference and flavor avoidance but does not produce flavor aversion in rats," *Behavioral Neuroscience*, vol. 110, pp. 503–508, 1996.

[122] J. C. Martin and E. H. Ellinwood, "Conditioned aversion to a preferred solution following methamphetamine injections," *Psychopharmacologia*, vol. 29, pp. 253–261, 1973.

[123] H. N. Manke, K. H. Nelson, A. Vlachos et al., "Assessment of aversive effects of methylone in male and female Sprague-Dawley rats: conditioned taste avoidance, body temperature and activity/stereotypies," *Neurotoxicology and Teratology*, vol. 86, article 106977, 2021.

[124] A. L. Riley and D. A. Zellner, "Methylphenidate-induced conditioned taste aversions: an index of toxicity," *Physiological Psychology*, vol. 6, pp. 354–358, 1978.

[125] F. Etscorn, "Sucrose aversions in mice as a result of injected nicotine or passive tobacco smoke inhalation," *Bulletin of the Psychonomic Society*, vol. 15, pp. 54–56, 1980.

[126] O. Burešová and J. Bureš, "Post-ingestion interference with brain function prevents attenuation of neophobia in rats," *Behavioural Brain Research*, vol. 1, pp. 299–312, 1980.

[127] H. Cappell and A. E. LeBlanc, "Gustatory avoidance conditioning by drugs of abuse," in *Food aversion learning*, N. W. Milgram, L. Krames, and T. M. Alloway, Eds., pp. 133–167, Springer, Boston, MA, 1977.

[128] E. Gamzu, "The multifaceted nature of taste aversion inducing agents: is there a single common factor?," in *Learning Mechanisms in Food Selection*, L. Barker, M. Best, and M. Domjan, Eds., pp. 477–510, Baylor University Press, Waco, TX, 1977.

[129] W. J. McBride, J. M. Murphy, and S. Ikemoto, "Localization of brain reinforcement mechanisms: Intracranial self-administration and intracranial place-conditioning studies," *Behavioural Brain Research*, vol. 101, pp. 129–152, 1999.

[130] A. L. Riley and L. L. Baril, "Conditioned taste aversions: a bibliography," *Animal Learning & Behavior*, vol. 4, pp. 1S–13S, 1976.

[131] S. Klosterhalfen and W. Klosterhalfen, "Conditioned taste aversion and traditional learning," *Psychological Research*, vol. 47, no. 2, pp. 71–94, 1985.

[132] P. S. Grigson and R. C. Twining, "Cocaine-induced suppression of saccharin intake: a model of drug-induced devaluation of natural rewards," *Behavioral Neuroscience*, vol. 116, pp. 321–333, 2002.

[133] L. H. Sellings, G. Baharnouri, L. E. McQuade, and P. B. Clarke, "Rewarding and aversive effects of nicotine are segregated within the nucleus accumbens," *European Journal of Neuroscience*, vol. 28, pp. 342–352, 2008.

[134] R. C. Twining, M. Bolan, and P. S. Grigson, "Yoked delivery of cocaine is aversive and protects against the motivation for drug in rats," *Behavioral Neuroscience*, vol. 123, pp. 913–925, 2009.

[135] A. M. Cason and P. S. Grigson, "Prior access to a sweet is more protective against cocaine self-administration in female rats than in male rats," *Physiology & Behavior*, vol. 112-113, pp. 96–103, 2013.

[136] R. M. Carelli and E. A. West, "When a good taste turns bad: neural mechanisms underlying the emergence of negative affect and associated natural reward devaluation by cocaine," *Neuropharmacology*, vol. 76, pp. 360–369, 2014.

[137] R. Wise, P. Yokel, and H. De Wit, "Both positive reinforcement and conditioned aversion from amphetamine and from apomorphine in rats," *Science*, vol. 191, pp. 1273-1274, 1976.

[138] A. Ettenberg and T. D. Geist, "Animal model for investigating the anxiogenic effects of self-administered cocaine," *Psychopharmacology*, vol. 103, pp. 455–461, 1991.

[139] A. Ettenberg and T. D. Geist, "Qualitative and quantitative differences in the operant runway behavior of rats working for cocaine and heroin reinforcement," *Pharmacology Biochemistry and Behavior*, vol. 44, pp. 191–198, 1993.

[140] N. White, L. Sklar, and Z. Amit, "The reinforcing action of morphine and its paradoxical side effect," *Psychopharmacology*, vol. 52, pp. 63–66, 1977.

[141] A. Ettenberg, V. Fomenko, K. Kaganovsky, K. Shelton, and J. M. Wenzel, "On the positive and negative affective responses to cocaine and their relation to drug self-administration in rats," *Psychopharmacology*, vol. 232, pp. 2363–2375, 2015.

[142] A. Ettenberg, M. A. Raven, D. A. Danluck, and B. D. Necessary, "Evidence for opponent-process actions of intravenous cocaine," *Pharmacology Biochemistry and Behavior*, vol. 64, pp. 507–512, 1999.

[143] M. A. Reicher and E. W. Holman, "Location preference and flavor aversion reinforced by amphetamine in rats," *Animal Learning & Behavior*, vol. 5, pp. 343–346, 1977.

[144] H. E. King, A. Wakeford, W. Taylor, B. Wetzell, K. C. Rice, and A. L. Riley, "Sex differences in 3,4-methylenedioxypyrovalerone (MDPV)-induced taste avoidance and place preferences," *Pharmacology Biochemistry and Behavior*, vol. 137, pp. 16–22, 2015.

[145] K. H. Nelson, H. N. Manke, A. Imanalieva, K. C. Rice, and A. L. Riley, "Sex differences in α-pyrrolidinopentiophenone (α-PVP)-induced taste avoidance, place preference, hyperthermia and locomotor activity in rats," *Pharmacology Biochemistry and Behavior*, vol. 185, article 172762, 2019.

[146] K. H. Nelson, H. M. Manke, J. M. Bailey et al., "Ethanol preexposure differentially impacts the rewarding and aversive effects of α-pyrrolidinopentiophenone (α-PVP): implications for drug use and abuse," *Pharmacology Biochemistry and Behavior*, vol. 211, article 173286, 2021.

[147] C. A. Dannenhoffer and L. P. Spear, "Age differences in conditioned place preferences and taste aversions to nicotine," *Developmental Psychobiology*, vol. 58, pp. 660–666, 2016.

[148] J. E. Sherman, T. Roberts, S. E. Roskam, and E. W. Holman, "Temporal properties of the rewarding and aversive effects of amphetamine in rats," *Pharmacology Biochemistry and Behavior*, vol. 13, pp. 597–599, 1980.

[149] Y. C. Wang, A. C. W. Huang, and S. Hsiao, "Paradoxical simultaneous occurrence of amphetamine-induced conditioned taste aversion and conditioned place preference with the same single drug injection: a new "pre-and post-association" experimental paradigm," *Pharmacology Biochemistry and Behavior*, vol. 95, pp. 80–87, 2010.

[150] A. Verendeev and A. L. Riley, "Relationship between the rewarding and aversive effects of morphine and amphetamine in individual subjects," *Learning & Behavior*, vol. 39, pp. 399–408, 2011.

[151] J. E. Sherman, C. Pickman, A. Rice, J. C. Liebeskind, and E. W. Holman, "Rewarding and aversive effects of morphine: temporal and pharmacological properties," *Pharmacology Biochemistry and Behavior*, vol. 13, pp. 501–505, 1980.

[152] G. R. Simpson and A. L. Riley, "Morphine preexposure facilitates morphine place preference and attenuates morphine taste aversion," *Pharmacology Biochemistry and Behavior*, vol. 80, pp. 471–479, 2005.

[153] H. E. King and A. L. Riley, "A history of morphine-induced taste aversion learning fails to affect morphine-induced place preference conditioning in rats," *Learning & Behavior*, vol. 41, pp. 433–442, 2013.

[154] G. C. Loney, C. P. King, and P. J. Meyer, "Systemic nicotine enhances opioid self-administration and modulates the formation of opioid-associated memories partly through actions within the insular cortex," *Scientific Reports*, vol. 11, pp. 1–13, 2021.

[155] Y. C. Wang, W. C. Chiu, C. N. Cheng, C. Lee, and A. C. W. Huang, "Examination of neuroinflammatory cytokine interleukin-1 beta expression in the medial prefrontal cortex,

amygdala, and hippocampus for the paradoxical effects of reward and aversion induced by morphine," *Neuroscience Letters*, vol. 760, article 136076, 2021.

[156] C. W. Wu, C. Y. Ou, Y. H. Yu, Y. C. Yu, B. C. Shyu, and A. C. W. Huang, "Involvement of the Ventral Tegmental Area but Not Periaqueductal Gray Matter in the Paradoxical Rewarding and Aversive Effects of Morphine," *Behavioral Neuroscience*, vol. 135, pp. 762–770, 2021.

[157] Y. Yu, A. B. He, M. Liou et al., "The paradoxical effect hypothesis of abused drugs in a rat model of chronic morphine administration," *Journal of Clinical Medicine*, vol. 10, p. 3197, 2021.

[158] L. A. Mayer and L. A. Parker, "Rewarding and aversive properties of IP and SC cocaine: assessment by place and taste conditioning," *Psychopharmacology*, vol. 112, pp. 189–194, 1993.

[159] R. L. Pomfrey, T. A. Bostwick, B. B. Wetzell, and A. L. Riley, "Adolescent nicotine exposure fails to impact cocaine reward, aversion and self-administration in adult male rats," *Pharmacology Biochemistry and Behavior*, vol. 137, pp. 30–37, 2015.

[160] M. M. Clasen, T. V. Sanon, D. N. Kearns, T. L. Davidson, and A. L. Riley, "Ad libitum high fat diet consumption during adolescence and adulthood fails to impact the affective properties of cocaine in male Sprague-Dawley rats," *Experimental and Clinical Psychopharmacology*, vol. 28, pp. 438–448, 2020.

[161] A. B. H. He, Y. C. Chang, A. W. Y. Meng, and A. C. W. Huang, "Re-evaluation of the reward comparison hypothesis for alcohol abuse," *Behavioural Brain Research*, vol. 332, pp. 218–222, 2017.

[162] N. T. Brockwell, R. Eikelboom, and R. J. Beninger, "Caffeine-induced place and taste conditioning: production of dose-dependent preference and aversion," *Pharmacology Biochemistry and Behavior*, vol. 38, pp. 513–517, 1991.

[163] P. G. Roma, W. W. Flint, J. D. Higley, and A. L. Riley, "Assessment of the aversive and rewarding effects of alcohol in Fischer and Lewis rats," *Psychopharmacology*, vol. 189, pp. 187–199, 2006.

[164] A. G. Wakeford, S. M. Flax, R. L. Pomfrey, and A. L. Riley, "Adolescent delta-9-tetrahydrocannabinol (THC) exposure fails to affect THC-induced place and taste conditioning in adult male rats," *Pharmacology Biochemistry and Behavior*, vol. 140, pp. 75–81, 2016.

[165] B. J. Hempel, A. G. Wakeford, M. M. Clasen, M. A. Friar, and A. L. Riley, "Delta-9-tetrahydrocannabinol (THC) history fails to affect THC's ability to induce place preferences in rats," *Pharmacology Biochemistry and Behavior*, vol. 144, pp. 1–6, 2016.

[166] A. L. Riley, "The paradox of drug taking: the role of the aversive effects of drugs," *Physiology & Behavior*, vol. 103, pp. 69–78, 2011.

[167] C. M. Davis, "Animal models of drug abuse: place and taste conditioning," in *Animal Models for the Study of Human Disease*, M. P. Conn, Ed., pp. 681–707, Academic Press, Cambridge, MA, 2013.

[168] M. J. Shram, D. Funk, Z. Li, and A. D. Lê, "Periadolescent and adult rats respond differently in tests measuring the rewarding and aversive effects of nicotine," *Psychopharmacology*, vol. 186, pp. 201–208, 2006.

[169] B. T. Lett, "Repeated exposures intensify rather than diminish the rewarding effects of amphetamine, morphine, and cocaine," *Psychopharmacology*, vol. 98, pp. 357–362, 1989.

[170] T. S. Shippenberg, C. H. Heidbreder, and A. Lefevour, "Sensitization to the conditioned rewarding effects of morphine: pharmacology and temporal characteristics," *European Journal of Pharmacology*, vol. 299, pp. 33–39, 1996.

[171] H. E. King, B. Wetzell, K. C. Rice, and A. L. Riley, "An assessment of MDPV-induced place preference in adult Sprague-Dawley rats," *Drug and Alcohol Dependence*, vol. 146, pp. 116–119, 2015.

[172] H. I. Risca, J. D. Zuarth-Gonzalez, and L. E. Baker, "Conditioned place preference following concurrent treatment with 3, 4-methylenedioxypyrovalerone (MDPV) and methamphetamine in male and female Sprague-Dawley rats," *Pharmacology Biochemistry and Behavior*, vol. 198, article 173032, 2020.

[173] M. B. Gatch, S. B. Dolan, and M. J. Forster, "Comparative behavioral pharmacology of three pyrrolidine-containing synthetic cathinone derivatives," *Journal of Pharmacology and Experimental Therapeutics*, vol. 354, pp. 103–110, 2015.

[174] J. A. Marusich, T. W. Lefever, B. E. Blough, B. F. Thomas, and J. L. Wiley, "Pharmacological effects of methamphetamine and alpha-PVP vapor and injection," *Neurotoxicology*, vol. 55, pp. 83–91, 2016.

[175] J. B. Bedingfield, D. A. King, and F. A. Holloway, "Cocaine and caffeine: conditioned place preference, locomotor activity, and additivity," *Pharmacology Biochemistry and Behavior*, vol. 61, pp. 291–296, 1998.

[176] A. L. Riley, C. M. Davis, and P. G. Roma, "Strain differences in taste aversion learning: Implications for animal models of drug abuse," in *Conditioned Taste Aversion: Behavioral and Neural Processes*, S. Reilly and T. R. Schachtman, Eds., pp. 226–261, Oxford University Press, New York, NY, 2009.

[177] A. L. Riley, B. J. Hempel, and M. M. Clasen, "Sex as a biological variable: drug use and abuse," *Physiology & Behavior*, vol. 187, pp. 79–96, 2018.

[178] I. P. Stolerman and G. D. D'Mello, "Oral self-administration and the relevance of conditioned taste aversions," in *Advances in Behavioral Pharmacology*, T. Thompson, P. B. Dews, and W. A. McKim, Eds., pp. 169–214, Elsevier, Amsterdam, Netherlands, 1981.

[179] A. Ettenberg, "The runway model of drug self-administration," *Pharmacology Biochemistry and Behavior*, vol. 91, pp. 271–277, 2009.

[180] S. D. Turenne, C. Miles, L. A. Parker, and S. Siegel, "Individual differences in reactivity to the rewarding/aversive properties of drugs: assessment by taste and place conditioning," *Pharmacology Biochemistry and Behavior*, vol. 53, pp. 511–516, 1996.

[181] J. A. Chester, F. O. Risinger, and C. L. Cunningham, "Ethanol reward and aversion in mice bred for sensitivity to ethanol withdrawal," *Alcoholism: Clinical and Experimental Research*, vol. 22, pp. 468–473, 1998.

[182] D. V. Gauvin, T. J. Baird, and R. J. Briscoe, "Differential development of behavioral tolerance and the subsequent hedonic effects of alcohol in AA and ANA rats," *Psychopharmacology*, vol. 151, pp. 335–343, 2000.

[183] J. M. Wheeler, C. Reed, S. Burkhart-Kasch et al., "Genetically correlated effects of selective breeding for high and low methamphetamine consumption," *Genes, Brain and Behavior*, vol. 8, pp. 758–771, 2009.

[184] W. S. Hyatt and W. E. Fantegrossi, "Δ9-THC exposure attenuates aversive effects and reveals appetitive effects of K2/

spice constituent JWH-018 in mice," *Behavioural Pharmacology*, vol. 25, pp. 253–257, 2014.

[185] K. G. Hill, H. Alva, Y. A. Blednov, and C. L. Cunningham, "Reduced ethanol-induced conditioned taste aversion and conditioned place preference in GIRK2 null mutant mice," *Psychopharmacology*, vol. 169, pp. 108–114, 2003.

[186] X. Li, B. J. Hempel, H. J. Yang et al., "Dissecting the role of CB1 and CB2 receptors in cannabinoid reward versus aversion using transgenic CB1-and CB2-knockout mice," *European Neuropsychopharmacology*, vol. 43, pp. 38–51, 2021.

[187] W. L. Isaac, A. J. Nonneman, J. Neisewander, T. Landers, and M. T. Bardo, "Prefrontal cortex lesions differentially disrupt cocaine-reinforced conditioned place preference but not conditioned taste aversion," *Behavioral Neuroscience*, vol. 103, pp. 345–355, 1989.

[188] T. M. Mosher, J. G. Smith, and A. J. Greenshaw, "Aversive stimulus properties of the 5-HT2C receptor agonist WAY 161503 in rats," *Neuropharmacology*, vol. 51, pp. 641–650, 2006.

[189] F. O. Risinger, P. A. Freeman, P. Greengard, and A. A. Fienberg, "Motivational effects of ethanol in DARPP-32 knockout mice," *The Journal of Neuroscience*, vol. 21, pp. 340–348, 2001.

[190] Y. A. Blednov, J. M. Benavidez, C. Geil, S. Perra, H. Morikawa, and R. Harris, "Activation of inflammatory signaling by lipopolysaccharide produces a prolonged increase of voluntary alcohol intake in mice," *Brain, Behavior, and Immunity*, vol. 25, pp. S92–S105, 2011.

[191] B. M. Rabin, J. A. Joseph, and B. Shukitt-Hale, "Long-term changes in amphetamine-induced reinforcement and aversion in rats following exposure to ^{56}Fe particle," *Advances in Space Research*, vol. 31, pp. 127–133, 2003.

[192] A. Bechara, K. A. Zito, and D. Van Der Kooy, "Peripheral receptors mediate the aversive conditioning effects of morphine in the rat," *Pharmacology Biochemistry and Behavior*, vol. 28, pp. 219–225, 1987.

[193] A. B. H. He, C. L. Huang, A. Kozłowska et al., "Involvement of neural substrates in reward and aversion to methamphetamine addiction: testing the reward comparison hypothesis and the paradoxical effect hypothesis of abused drugs," *Neurobiology of Learning and Memory*, vol. 166, article 107090, 2019.

[194] S. Ghozland, H. W. Matthes, F. Simonin, D. Filliol, B. L. Kieffer, and R. Maldonado, "Motivational effects of cannabinoids are mediated by μ-opioid and κ-opioid receptors," *The Journal of Neuroscience*, vol. 22, pp. 1146–1154, 2002.

[195] N. R. Gubner, C. Reed, C. S. McKinnon, and T. J. Phillips, "Unique genetic factors influence sensitivity to the rewarding and aversive effects of methamphetamine versus cocaine," *Behavioural Brain Research*, vol. 256, pp. 420–427, 2013.

[196] J. Garcia and D. J. Kimeldorf, "Temporal relationship within the conditioning of a saccharine aversion through radiation exposure," *Journal of Comparative and Physiological Psychology*, vol. 50, pp. 180–183, 1957.

[197] J. Garcia and D. J. Kimeldorf, "Some factors which influence radiation-conditioned behavior of rats," *Radiation Research*, vol. 12, pp. 719–727, 1960.

[198] A. J. Goudie, I. P. Stolerman, C. Demellweek, and G. D. D'Mello, "Does conditioned nausea mediate drug-induced conditioned taste aversion?," *Psychopharmacology*, vol. 78, pp. 277–281, 1982.

[199] L. A. Parker, S. A. Rana, and C. L. Limebeer, "Conditioned nausea in rats: assessment by conditioned disgust reactions, rather than conditioned taste avoidance," *Canadian Journal of Experimental Psychology*, vol. 62, pp. 198–209, 2008.

[200] B. J. Hempel, M. M. Clasen, K. H. Nelson, C. J. Woloshchuk, and A. L. Riley, "An assessment of concurrent cannabidiol and Δ^9-tetrahydrocannabinol administration in place aversion and taste avoidance conditioning," *Experimental and Clinical Psychopharmacology*, vol. 26, pp. 205–213, 2018.

[201] L. M. Barker, J. C. Smith, and E. M. Suarez, "Sickness and the backward conditioning of taste aversions," in *Learning Mechanisms in Food Selection*, L. M. Barker, M. Best, and M. Domjan, Eds., pp. 533–553, Baylor University Press, Waco, TX, 1977.

[202] L. A. Parker, "Conditioned flavor avoidance and conditioned gaping: rat models of conditioned nausea," *European Journal of Pharmacology*, vol. 722, pp. 122–133, 2014.

[203] H. J. Grill and R. Norgren, "The taste reactivity test. I. Mimetic responses to gustatory stimuli in neurologically normal rats," *Brain Research*, vol. 143, pp. 263–279, 1978.

[204] L. A. Schier, K. M. Hyde, and A. C. Spector, "Conditioned taste aversion versus avoidance: a re-examination of the separate processes hypothesis," *Plos One*, vol. 14, article e0217458, 2019.

[205] L. A. Parker, "Nonconsummatory and consummatory behavioral CRs elicited by lithium-and amphetamine-paired flavors," *Learning and Motivation*, vol. 13, pp. 281–303, 1982.

[206] L. A. Parker, "Emetic drugs produce conditioned rejection reactions in the taste reactivity test," *Journal of Psychophysiology*, vol. 12, pp. 3–13, 1998.

[207] L. A. Parker, "Taste-reactivity responses elicited by reinforcing drugs: a dose-response analysis," *Behavioral Neuroscience*, vol. 105, pp. 955–964, 1991.

[208] L. A. Parker, "Rewarding drugs produce taste avoidance, but not taste aversion," *Neuroscience & Biobehavioral Reviews*, vol. 19, no. 1, pp. 143–151, 1995.

[209] L. A. Parker, "Taste avoidance and taste aversion: evidence for two different processes," *Animal Learning & Behavior*, vol. 31, pp. 165–172, 2003.

[210] C. L. Limebeer and L. A. Parker, "The antiemetic drug ondansetron intereferes with lithium-induced conditioned rejection reactions, but not lithium induced taste avoidance in rats," *Journal of Experimental Psychology: Animal Behavior Processes*, vol. 26, pp. 371–384, 2000.

[211] J. Y. Lin, J. Arthurs, L. R. Amodeo, and S. Reilly, "Reduced palatability in drug-induced taste aversion: I. Variations in the initial value of the conditioned stimulus," *Behavioral Neuroscience*, vol. 126, pp. 423–432, 2012.

[212] J. Y. Lin, J. Arthurs, and S. Reilly, "Reduced palatability in pain-induced conditioned taste aversions," *Physiology & Behavior*, vol. 119, pp. 79–85, 2013.

[213] J. Y. Lin, J. Arthurs, and S. Reilly, "Conditioned taste aversion, drugs of abuse and palatability," *Neuroscience & Biobehavioral Reviews*, vol. 45, pp. 28–45, 2014.

[214] J. Y. Lin, J. Arthurs, and S. Reilly, "Conditioned taste aversions: from poisons to pain to drugs of abuse," *Psychonomic Bulletin & Review*, vol. 24, pp. 335–351, 2017.

[215] J. Y. Lin, J. Arthurs, and S. Reilly, "Anesthesia-inducing drugs also induce conditioned taste aversions," *Physiology & Behavior*, vol. 177, pp. 247–251, 2017.

[216] D. M. Dwyer, R. A. Boakes, and A. J. Hayward, "Reduced palatability in lithium-and activity-based, but not in amphetamine-based, taste aversion learning," *Behavioral Neuroscience*, vol. 122, pp. 1051–1060, 2008.

[217] D. M. Dwyer, "Licking and liking: the assessment of hedonic responses in rodents," *Quarterly Journal of Experimental Psychology*, vol. 65, pp. 371–394, 2012.

[218] T. Hunt and Z. Amit, "Conditioned taste aversion induced by self-administered drugs: paradox revisited," *Neuroscience & Biobehavioral Reviews*, vol. 11, pp. 107–130, 1987.

[219] A. L. Riley and G. R. Simpson, "The attenuating effects of drug preexposure on taste aversion conditioning: generality, experimental parameters, underlying mechanisms, and implications for drug use and abuse," in *Handbook of Contemporary Learning Theories*, R. R. Mowrer and S. B. Klein, Eds., pp. 505–559, Lawrence Erlbaum Associates Publishers, NJ, 2001.

[220] C. L. Cunningham, D. M. Hawks, and D. R. Niehus, "Role of hypothermia in ethanol-induced conditioned taste aversion," *Psychopharmacology*, vol. 95, pp. 318–322, 1988.

[221] R. A. Wheeler, R. C. Twining, J. L. Jones, J. M. Slater, P. S. Grigson, and R. M. Carelli, "Behavioral and electrophysiological indices of negative affect predict cocaine self-administration," *Neuron*, vol. 57, pp. 774–785, 2008.

[222] E. M. Colechio, D. N. Alexander, C. G. Imperio, K. Jackson, and P. S. Grigson, "Once is too much: early development of the opponent process in taste reactivity behavior is associated with later escalation of cocaine self-administration in rats," *Brain Research Bulletin*, vol. 138, pp. 88–95, 2018.

[223] K. G. Guenther, C. E. Wideman, E. M. Rock, C. L. Limebeer, and L. A. Parker, "Conditioned aversive responses produced by delayed, but not immediate, exposure to cocaine and morphine in male Sprague-Dawley rats," *Psychopharmacology*, vol. 235, pp. 3315–3327, 2018.

[224] L. A. Parker, C. L. Limebeer, and S. A. Rana, "Conditioned disgust, but not conditioned taste avoidance, may reflect conditioned nausea in rats," in *Conditioned Taste Aversions: Behavioral and Neural Processes*, S. Reilly and T. R. Schachtman, Eds., pp. 92–113, Oxford University Press, New York, NY, 2009.

[225] G. Takács, B. Lukáts, S. Papp, C. Szalay, and Z. Karádi, "Taste reactivity alterations after IL-1β microinjection into the ventromedial hypothalamic nucleus of the rat," *Neuroscience Research*, vol. 62, pp. 118–122, 2008.

[226] T. M. Barney, A. S. Vore, A. Gano, J. E. Mondello, and T. Deak, "The influence of central interleukin-6 on behavioral changes associated with acute alcohol intoxication in adult male rats," *Alcohol*, vol. 79, pp. 37–45, 2019.

[227] J. D. Patel and I. S. Ebenezer, "The effect of intraperitoneal administration of leptin on short-term food intake in rats," *European Journal of Pharmacology*, vol. 580, pp. 143–152, 2008.

[228] K. Abegg, L. Bernasconi, M. Hutter et al., "Ghrelin receptor inverse agonists as a novel therapeutic approach against obesity-related metabolic disease," *Diabetes, Obesity and Metabolism*, vol. 19, pp. 1740–1750, 2017.

[229] J. A. Rodriguez, J. A. Fehrentz, J. Martinez, K. B. H. Salah, and P. J. Wellman, "The GHR-R antagonist JMV 2959 neither induces malaise nor alters the malaise property of LiCl in the adult male rat," *Physiology & Behavior*, vol. 183, pp. 46–48, 2018.

[230] S. N. Gartner, F. Aidney, A. Klockars et al., "Intragastric preloads of L-tryptophan reduce ingestive behavior via oxytocinergic neural mechanisms in male mice," *Appetite*, vol. 125, pp. 278–286, 2018.

[231] M. P. Gillum, D. Zhang, X. M. Zhang et al., "N-Acylphosphatidylethanolamine, a gut-derived circulating factor induced by fat ingestion, inhibits food intake," *Cell*, vol. 135, pp. 813–824, 2008.

[232] K. Proulx, D. Cota, T. R. Castañeda et al., "Mechanisms of oleoylethanolamide-induced changes in feeding behavior and motor activity," *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, vol. 289, pp. R729–R737, 2005.

[233] P. S. Grigson, "Conditioned taste aversions and drugs of abuse: a reinterpretation," *Behavioral Neuroscience*, vol. 111, pp. 129–136, 1997.

[234] A. C. W. Huang and S. Hsiao, "Re-examination of amphetamine-induced conditioned suppression of tastant intake in rats: the task-dependent drug effects hypothesis," *Behavioral Neuroscience*, vol. 122, pp. 1207–1216, 2008.

[235] A. C. W. Huang, C. C. Wang, and S. Wang, "Examinations of the reward comparison hypothesis: the modulation of gender and footshock," *Physiology & Behavior*, vol. 151, pp. 129–138, 2015.

[236] C. L. Cunningham, C. M. Gremel, and P. A. Groblewski, "Genetic influences on conditioned taste aversion," in *Conditioned Taste Aversion: Behavioral and Neural Processes*, S. Reilly and T. R. Schachtman, Eds., pp. 387–421, Oxford University Press, New York, NY, 2009.

[237] D. B. Newlin and R. M. Renton, "High risk groups often have higher levels of alcohol response than low risk: the other side of the coin," *Alcoholism: Clinical and Experimental Research*, vol. 34, pp. 199–202, 2010.

[238] M. A. Schuckit, "Low level of response to alcohol as a predictor of future alcoholism," *American Journal of Psychiatry*, vol. 151, pp. 184–189, 1994.

[239] H. J. Edenberg, "The genetics of alcohol metabolism: role of alcohol dehydrogenase and aldehyde dehydrogenase variants," *Alcohol Research & Health*, vol. 30, pp. 5–13, 2007.

[240] J. C. Crabbe, "Genetic contributions to addiction," *Annual Review of Psychology*, vol. 53, pp. 435–462, 2002.

[241] R. L. Elkins, "Separation of taste-aversion-prone and taste-aversion-resistant rats through selective breeding: implications for individual differences in conditionability and aversion-therapy alcoholism treatment," *Behavioral Neuroscience*, vol. 100, pp. 121–124, 1986.

[242] R. L. Elkins, P. A. Walters, and T. E. Orr, "Continued development and unconditioned stimulus characterization of selectively bred lines of taste aversion prone and resistant rats," *Alcoholism: Clinical and Experimental Research*, vol. 16, pp. 928–934, 1992.

[243] T. E. Orr, J. L. Whitford-Stoddard, and R. L. Elkins, "Taste-aversion-prone (TAP) rats and taste-aversion-resistant (TAR) rats differ in ethanol self-administration, but not in ethanol clearance or general consumption," *Alcohol*, vol. 33, pp. 1–7, 2004.

[244] R. L. Elkins, T. E. Orr, J. L. Rausch et al., "Cocaine-induced expression differences in glutamate receptor subunits and transporters in amygdalae of taste aversion-prone and taste aversion-resistant rats," *Annals of the New York Academy of Sciences*, vol. 1003, pp. 381–385, 2003.

[245] S. H. Hobbs, P. A. Walters, E. F. Shealy, and R. L. Elkins, "Radial-maze learning by lines of taste-aversion-prone and taste-aversion-resistant rats," *Bulletin of the Psychonomic Society*, vol. 31, pp. 171–174, 1993.

[246] T. E. Orr, P. A. Walters, and R. L. Elkins, "Differences in free-choice ethanol acceptance between taste aversion-prone and taste aversion-resistant rats," *Alcoholism: Clinical and Experimental Research*, vol. 21, pp. 1491–1496, 1997.

[247] D. S. Cannon and L. E. Carrell, "Rat strain differences in ethanol self-administration and taste aversion learning," *Pharmacology Biochemistry and Behavior*, vol. 28, pp. 57–63, 1987.

[248] S. Cailhol and P. Mormède, "Conditioned taste aversion and alcohol drinking: strain and gender differences," *Journal of Studies on Alcohol*, vol. 63, pp. 91–99, 2002.

[249] J. Broadbent, K. J. Muccino, and C. L. Cunningham, "Ethanol-induced conditioned taste aversion in 15 inbred mouse strains," *Behavioral Neuroscience*, vol. 116, pp. 138–148, 2002.

[250] C. L. Cunningham, "Genetic relationships between ethanol-induced conditioned place aversion and other ethanol phenotypes in 15 inbred mouse strains," *Brain Sciences*, vol. 9, pp. 209–229, 2019.

[251] C. L. Cunningham, "Genetic relationship between ethanol-induced conditioned place preference and other ethanol phenotypes in 15 inbred mouse strains," *Behavioral Neuroscience*, vol. 128, pp. 430–445, 2014.

[252] S. Shabani, C. S. McKinnon, C. Reed, C. L. Cunningham, and T. J. Phillips, "Sensitivity to rewarding or aversive effects of methamphetamine determines methamphetamine intake," *Genes, Brain and Behavior*, vol. 10, pp. 625–636, 2011.

[253] S. Shabani, C. S. McKinnon, C. L. Cunningham, and T. J. Phillips, "Profound reduction in sensitivity to the aversive effects of methamphetamine in mice bred for high methamphetamine intake," *Neuropharmacology*, vol. 62, pp. 1134–1141, 2012.

[254] D. Lancellotti, B. M. Bayer, J. R. Glowa, R. A. Houghtling, and A. L. Riley, "Morphine-induced conditioned taste aversions in the LEW/N and F344/N rat strains," *Pharmacology Biochemistry and Behavior*, vol. 68, pp. 603–610, 2001.

[255] C. M. Davis, K. C. Rice, and A. L. Riley, "Opiate-agonist induced taste aversion learning in the Fischer 344 and Lewis inbred rat strains: evidence for differential mu opioid receptor activation," *Pharmacology Biochemistry and Behavior*, vol. 93, pp. 397–405, 2009.

[256] C. M. Davis, J. L. Cobuzzi, and A. L. Riley, "Assessment of the aversive effects of peripheral mu opioid receptor agonism in Fischer 344 and Lewis rats," *Pharmacology Biochemistry and Behavior*, vol. 101, pp. 181–186, 2012.

[257] K. A. Pescatore, J. R. Glowa, and A. L. Riley, "Strain differences in the acquisition of nicotine-induced conditioned taste aversion," *Pharmacology Biochemistry and Behavior*, vol. 82, pp. 751–757, 2005.

[258] M. M. Foynes and A. L. Riley, "Lithium-chloride-induced conditioned taste aversions in the Lewis and Fischer 344 rat strains," *Pharmacology Biochemistry and Behavior*, vol. 79, pp. 303–308, 2004.

[259] J. R. Glowa, A. E. Shaw, and A. L. Riley, "Cocaine-induced conditioned taste aversions: comparisons between effects in LEW/N and F344/N rat strains," *Psychopharmacology*, vol. 114, pp. 229–232, 1994.

[260] J. M. Vishwanath, A. G. Desko, and A. L. Riley, "Caffeine-induced taste aversions in Lewis and Fischer rat strains: differential sensitivity to the aversive effects of drugs," *Pharmacology Biochemistry and Behavior*, vol. 100, pp. 66–72, 2011.

[261] P. G. Roma, C. M. Davis, and A. L. Riley, "Effects of cross-fostering on cocaine-induced conditioned taste aversions in Fischer and Lewis rats," *Developmental Psychobiology*, vol. 49, pp. 172–179, 2007.

[262] M. Gomez-Serrano, L. Tonelli, S. Listwak, E. Sternberg, and A. L. Riley, "Effects of cross fostering on open-field behavior, acoustic startle, lipopolysaccharide-induced corticosterone release, and body weight in Lewis and Fischer rats," *Behavior Genetics*, vol. 31, pp. 427–436, 2001.

[263] M. A. Gomez-Serrano, E. M. Sternberg, and A. L. Riley, "Maternal behavior in F344/N and LEW/N rats: effects on carrageenan-induced inflammatory reactivity and body weight," *Physiology & Behavior*, vol. 75, pp. 493–505, 2002.

[264] M. C. Camp, M. Feyder, J. Ihne et al., "A novel role for PSD-95 in mediating ethanol intoxication, drinking and place preference," *Addiction Biology*, vol. 16, pp. 428–439, 2011.

[265] Y. A. Blednov, J. M. Benavidez, M. Black, J. Mayfield, and R. A. Harris, "Role of interleukin-1 receptor signaling in the behavioral effects of ethanol and benzodiazepines," *Neuropharmacology*, vol. 95, pp. 309–320, 2015.

[266] R. Legastelois, E. Darcq, S. A. Wegner, P. J. Lombroso, and D. Ron, "Striatal-enriched protein tyrosine phosphatase controls responses to aversive stimuli: implication for ethanol drinking," *Plos One*, vol. 10, article e0127408, 2015.

[267] T. Isse, T. Oyama, K. Kitagawa et al., "Diminished alcohol preference in transgenic mice lacking aldehyde dehydrogenase activity," *Pharmacogenetics and Genomics*, vol. 12, pp. 621–626, 2002.

[268] T. Isse, K. Matsuno, T. Oyama, K. Kitagawa, and T. Kawamoto, "Aldehyde dehydrogenase 2 gene targeting mouse lacking enzyme activity shows high acetaldehyde level in blood, brain, and liver after ethanol gavages," *Alcoholism: Clinical & Experimental Research*, vol. 29, pp. 1959–1964, 2005.

[269] N. L. Schramm-Sapyta, Q. D. Walker, J. M. Caster, E. D. Levin, and C. M. Kuhn, "Are adolescents more vulnerable to drug addiction than adults? Evidence from animal models," *Psychopharmacology*, vol. 206, pp. 1–21, 2009.

[270] L. P. Spear, "Adolescents and alcohol: acute sensitivities, enhanced intake, and later consequences," *Neurotoxicology and Teratology*, vol. 41, pp. 51–59, 2014.

[271] M. M. Clasen, T. V. Sanon, B. J. Hempel et al., "Ad-libitum high fat diet consumption during adolescence and adulthood impacts the intravenous self-administration of cocaine in male Sprague-Dawley rats," *Experimental and Clinical Psychopharmacology*, vol. 28, pp. 32–43, 2020.

[272] A. L. Riley, M. M. Clasen, and M. A. Friar, "Conditioned taste avoidance drug discrimination procedure: assessments and applications," *Current Topics in Behavioral Neurosciences*, vol. 39, pp. 297–317, 2018.

[273] M. M. Clasen, A. L. Riley, and T. L. Davidson, "Hippocampal-dependent inhibitory learning and memory processes in the control of eating and drug taking," *Current Pharmaceutical Design*, vol. 26, pp. 2334–3352, 2020.

[274] L. D. Hill-Bowen, M. C. Riedel, R. Poudel et al., "The cue-reactivity paradigm: an ensemble of networks driving attention and cognition when viewing drug and natural reward-

related stimuli," *Neuroscience & Biobehavioral Reviews*, vol. 130, pp. 201–213, 2021.

[275] H. Ekhtiari, P. Nasseri, F. Yavari, A. Mokri, and J. Monterosso, "Neuroscience of drug craving for addiction medicine: from circuits to therapies," *Progress in Brain Research*, vol. 223, pp. 115–141, 2016.

[276] M. M. Torregrossa and J. R. Taylor, "Neuroscience of learning and memory for addiction medicine: from habit formation to memory reconsolidation," *Progress in Brain Research*, vol. 223, pp. 91–113, 2016.

[277] C. J. Perry, I. Zbukvic, J. H. Kim, and A. J. Lawrence, "Role of cues and contexts on drug-seeking behaviour," *British Journal of Pharmacology*, vol. 171, pp. 4636–4672, 2014.

[278] L. V. Panlilio, E. B. Thorndike, and C. W. Schindler, "A stimulus-control account of regulated drug intake in rats," *Psychopharmacology*, vol. 196, pp. 441–450, 2008.

[279] S. Jones, A. Hyde, and T. L. Davidson, "Reframing appetitive reinforcement learning and reward valuation as effects mediated by hippocampal-dependent behavioral inhibition," *Nutrition Research*, vol. 79, pp. 1–12, 2020.

[280] V. L. Tsibulsky and A. B. Norman, "Satiety threshold during maintained cocaine self-administration in outbred mice," *Neuroreport*, vol. 12, pp. 325–328, 2001.

[281] S. Trask, E. A. Thrailkill, and M. E. Bouton, "Occasion setting, inhibition, and the contextual control of extinction in Pavlovian and instrumental (operant) learning," *Behavioural Processes*, vol. 137, pp. 64–72, 2017.

[282] K. A. Carr, T. O. Daniel, H. Lin, and L. H. Epstein, "Reinforcement pathology and obesity," *Current Drug Abuse Reviews*, vol. 4, pp. 190–196, 2011.

[283] T. L. Davidson, A. L. Tracy, L. A. Schier, and S. E. Swithers, "A view of obesity as a learning and memory disorder," *Journal of Experimental Psychology: Animal Learning and Cognition*, vol. 40, pp. 261–279, 2014.

[284] S. Dohle, K. Diel, and W. Hofmann, "Executive functions and the self-regulation of eating behavior: a review," *Appetite*, vol. 124, pp. 4–9, 2018.

[285] R. L. Balster, "Drugs as chemical stimuli," *Transduction Mechanisms of Drug Stimuli*, vol. 4, pp. 3–11, 1988.

[286] F. C. Colpaert, "Drug discrimination in neurobiology," *Pharmacology Biochemistry and Behavior*, vol. 64, no. 2, pp. 337–345, 1999.

[287] J. H. Porter and A. J. Prus, "Discriminative stimulus properties of atypical and typical antipsychotic drugs: a review of preclinical studies," *Psychopharmacology*, vol. 203, pp. 279–294, 2009.

[288] J. R. Troisi and N. L. Michaud, "Can the discriminative stimulus effects of nicotine function concurrently as modulatory opponents in operant and Pavlovian occasion setting paradigms in rats?," *Behavioural Processes*, vol. 158, pp. 144–150, 2019.

[289] R. A. Glennon, T. U. Järbe, and J. Frankenheim, *Drug Discrimination: Applications to Drug Abuse Research. US Department of Health and Human Services, Public Health Service, Alcohol, Drug Abuse, and Mental Health Administration*, National Institute on Drug Abuse, Rockville, MD, 1991.

[290] A. L. Riley, "Use of drug discrimination learning in behavioral toxicology: classification and characterization of toxins," in *Neurotoxicology: Approaches and Methods*, L. Chang and W. Slikker, Eds., pp. 309–321, Academic Press, San Diego, CA, 1995.

[291] S. D. Grabus, S. T. Smurthwaite, and A. L. Riley, "Nalorphine's ability to substitute for morphine in a drug discrimination procedure is a function of training dose," *Pharmacology Biochemistry and Behavior*, vol. 63, pp. 481–488, 1999.

[292] Y. Awasaki, H. Nojima, and N. Nishida, "Application of the conditioned taste aversion paradigm to assess discriminative stimulus properties of psychostimulants in rats," *Drug and Alcohol Dependence*, vol. 118, no. 2-3, pp. 288–294, 2011.

[293] J. P. Mastropaolo, K. H. Moskowitz, R. J. Dacanay, and A. L. Riley, "Conditioned taste aversions as a behavioral baseline for drug discrimination learning: an assessment with phencyclidine," *Pharmacology Biochemistry and Behavior*, vol. 32, pp. 1–8, 1989.

[294] I. Lucki, "Rapid discrimination of the stimulus properties of 5-hydroxytryptamine agonists using conditioned taste aversion," *Journal of Pharmacology and Experimental Therapeutics*, vol. 247, pp. 1120–1127, 1988.

[295] T. V. Jaeger and R. F. Mucha, "A taste aversion model of drug discrimination learning: training drug and condition influence rate of learning, sensitivity and drug specificity," *Psychopharmacology*, vol. 100, pp. 145–150, 1990.

[296] D. M. Skinner, G. M. Martin, R. D. Howe, A. Pridgar, and D. van der Kooy, "Drug discrimination learning using a taste aversion paradigm: an assessment of the role of safety cues," *Learning and Motivation*, vol. 26, pp. 343–369, 1995.

[297] G. M. Martin, M. Gans, and D. van der Kooy, "Discriminative properties of morphine that modulate associations between tastes and lithium chloride," *Journal of Experimental Psychology: Animal Behavior Processes*, vol. 16, no. 1, pp. 56–68, 1990.

[298] S. Maren and W. Holt, "The hippocampus and contextual memory retrieval in Pavlovian conditioning," *Behavioural Brain Research*, vol. 110, no. 1-2, pp. 97–108, 2000.

[299] A. P. Maurer and L. Nadel, "The continuity of context: a role for the hippocampus," *Trends in Cognitive Sciences*, vol. 25, pp. 187–199, 2021.

[300] P. C. Holland and M. E. Bouton, "Hippocampus and context in classical conditioning," *Current Opinion in Neurobiology*, vol. 9, pp. 195–202, 1999.

[301] N. Hebben, S. Corkin, H. Eichenbaum, and K. Shedlack, "Diminished ability to interpret and report internal states after bilateral medial temporal resection: case H.M," *Behavioral Neuroscience*, vol. 99, pp. 1031–1039, 1985.

[302] P. Rozin, S. Dow, M. Moscovitch, and S. Rajaram, "What causes humans to begin and end a meal? A role for memory for what has been eaten, as evidenced by a study of multiple meal eating in amnesic patients," *Psychological Science*, vol. 9, pp. 392–396, 1998.

[303] S. Higgs, A. C. Williamson, P. Rotshtein, and G. W. Humphreys, "Sensory-specific satiety is intact in amnesics who eat multiple meals: research report," *Psychological Science*, vol. 19, pp. 623–628, 2008.

[304] T. L. Davidson and L. E. Jarrard, "A role for hippocampus in the utilization of hunger signals," *Behavioral and Neural Biology*, vol. 59, pp. 167–171, 1993.

[305] T. L. Davidson, S. E. Kanoski, K. Chan, D. J. Clegg, S. C. Benoit, and L. E. Jarrard, "Hippocampal lesions impair retention of discriminative responding based on energy state cues," *Behavioral Neuroscience*, vol. 124, pp. 97–105, 2010.

[306] Y. O. Henderson, G. P. Smith, and M. B. Parent, "Hippocampal neurons inhibit meal onset," *Hippocampus*, vol. 23, pp. 100–107, 2013.

[307] R. Hannapel, J. Ramesh, A. Ross, R. T. Lalumiere, A. G. Roseberry, and M. B. Parent, "Postmeal optogenetic inhibition of dorsal or ventral hippocampal pyramidal neurons increases future intake," *Eneuro*, vol. 6, pp. 1–16, 2019.

[308] S. B. Briggs, R. Hannapel, J. Ramesh, and M. B. Parent, "Inhibiting ventral hippocampal NMDA receptors and arc increases energy intake in male rats," *Learning & Memory*, vol. 28, pp. 187–194, 2021.

[309] T. L. Davidson, K. Chan, L. E. Jarrard, S. E. Kanoski, D. J. Clegg, and S. C. Benoit, "Contributions of the hippocampus and medial prefrontal cortex to energy and body weight regulation," *Hippocampus*, vol. 19, pp. 235–252, 2009.

[310] C. H. Sample, S. Jones, S. L. Hargrave, L. E. Jarrard, and T. L. Davidson, "Western diet and the weakening of the interoceptive stimulus control of appetitive behavior," *Behavioural Brain Research*, vol. 312, pp. 219–230, 2016.

[311] S. D. Glick and R. D. Cox, "Changes in morphine self-administration after tel-diencephalic lesions in rats," *Psychopharmacology*, vol. 57, pp. 283–288, 1978.

[312] R. M. Karlsson, D. M. Kircher, Y. Shaham, and P. O'Donnell, "Exaggerated cue-induced reinstatement of cocaine seeking but not incubation of cocaine craving in a developmental rat model of schizophrenia," *Psychopharmacology*, vol. 226, pp. 45–51, 2013.

[313] R. A. Chambers and D. W. Self, "Motivational responses to natural and drug rewards in rats with neonatal ventral hippocampal lesions: an animal model of dual diagnosis schizophrenia," *Neuropsychopharmacology*, vol. 27, pp. 889–905, 2002.

[314] S. K. Conroy, Z. Rodd, and R. A. Chambers, "Ethanol sensitization in a neurodevelopmental lesion model of schizophrenia in rats," *Pharmacology Biochemistry and Behavior*, vol. 86, pp. 386–394, 2007.

[315] A. M. Brady, R. D. Saul, and M. K. Wiest, "Selective deficits in spatial working memory in the neonatal ventral hippocampal lesion rat model of schizophrenia," *Neuropharmacology*, vol. 59, pp. 605–611, 2010.

[316] S. A. Berg, A. M. Sentir, B. S. Cooley, E. A. Engleman, and R. A. Chambers, "Nicotine is more addictive, not more cognitively therapeutic in a neurodevelopmental model of schizophrenia produced by neonatal ventral hippocampal lesions," *Addiction Biology*, vol. 19, pp. 1020–1031, 2014.

[317] T. L. Davidson, S. L. Hargrave, D. N. Kearns et al., "Cocaine impairs serial-feature negative learning and blood-brain barrier integrity," *Pharmacology Biochemistry and Behavior*, vol. 170, pp. 56–63, 2018.

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Research Article

Analysis of Gut Microbiota in Patients with Exacerbated Symptoms of Schizophrenia following Therapy with Amisulpride: A Pilot Study

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Evidence is mounting that the gut microbiome is related to the underlying pathogenesis of schizophrenia. However, effects of amisulpride on gut microbiota are poorly defined. This study was aimed at analyzing cytokines and fecal microbiota in patients with exacerbated symptoms of schizophrenia treated with amisulpride during four weeks of their hospital stay. In the present study, feces collected from patients with schizophrenia were analyzed using 16S rRNA pyrosequencing and bioinformatic analyses to ascertain gut microbiome composition and fasting peripheral blood cytokines. We found that patients undergoing treatment of schizophrenia with amisulpride had distinct changes in gut microbial composition at the genus level, increased levels of short-chain fatty acid-producing bacteria (*Dorea* and *Butyricicoccus*), and reduced levels of pathogenic bacteria (*Actinomyces* and *Porphyromonas*), but the level of *Desulfovibrio* was still high. We also found a significant downregulation of butanoate metabolism based on functional analysis of the microbiome. After treatment, elevated levels of interleukin- (IL-) 4 and decreased levels of IL-6 were found. Our findings extend prior work and suggest a possible pharmacological mechanism of amisulpride treatment for schizophrenia, which acts via mediation of the gut microbiome.

1. Introduction

Schizophrenia (SCZ) is a psychiatric disease associated with psychosis, thought disorders, alterations in drive, volition, and neurocognition, affecting approximately 0.5% to 1.0% of the population worldwide, while the prevalence of SCZ is 5.44 per 1000 in China [1]. The developmental pathophysiological mechanism of SCZ is quite complex and has not yet been clearly elucidated.

Interestingly, shreds of evidence show that the gut microbiome may play a critical role in the pathogenesis of SCZ, and the pathophysiology involved in SCZ may also be regulated via the "microbiota-gut-brain" (MGB) axis [2, 3]. The composition of gut microbiota and its metabolites has been implicated in the development of SCZ. Alterations of bacterial taxa have been observed in SCZ, especially in decreased relative amounts of short-chain fatty acid- (SCFA-) producing bacteria and elevated pathogenic bacteria [4, 5]. Fecal microbiota transplantation techniques have been used to explore the mechanism of SCZ, including whether the gut microbiome of patients with SCZ modulates neurochemistry and neurologic functioning in rodents. These techniques demonstrated that the gut microbiome

plays an important role in the development of SCZ [6, 7]. Additionally, gut-associated immune imbalances have also been demonstrated in patients with SCZ, particularly in relation to mucosal immunity [8], which is consistent with the developmental inflammatory hypothesis of explaining the pathogenesis of SCZ [9].

However, gut microbiota taxa are influenced by dietary habits, drugs, and environmental factors [10]. Our previous study revealed that antipsychotics can regulate metabolic and inflammatory abnormalities such as cytokines in patients with acutely exacerbated symptoms of schizophrenia [11]. Although drugs are a major therapeutic strategy for SCZ, many patients with SCZ have repeated episodes and experience medication side effects. A recent study found that olanzapine therapy in patients with SCZ altered gut microbiota including their metabolism [12]. However, the effect of amisulpride on gut microbiota has not been investigated in patients with schizophrenia, including whether the pharmacological mechanism of amisulpride treatment for SCZ acts via meditation of the gut microbiome.

Amisulpride is an effective, well-tolerated, and widely used dopamine D2 and D3 receptor antagonist for Chinese patients, which can improve SCZ symptoms effectively especially the negative symptoms of schizophrenia [13, 14]. Accordingly, we hypothesized that short-term therapy with amisulpride can ameliorate SCZ symptoms, mediated by the microbiome. The present study was aimed at investigating the microbiota composition of fecal samples as well as blood cytokines measured in a cohort of inpatients with SCZ who had acute exacerbated symptoms; the cohort's dietary intake and exposure to environmental factors were kept constant. The cohort was comprised of acutely exacerbated inpatients with SCZ, who were followed up for four weeks during amisulpride treatment.

2. Materials and Methods

2.1. Study Population. The Institutional Review Board of the Third Hospital of Quanzhou approved this study (IRB No. 2018001). All participants agreed to participate in this study and provided signed written informed consent before enrollment. Participants were recruited as inpatients at the Third Hospital of Quanzhou. The diagnosis of SCZ was based on Diagnostic and Statistical Manual of Mental Disorders (DSM) IV criteria. Participants were recruited when their symptoms were acutely exacerbated and treated with amisulpride (dosage from 400 to 1200 mg/kg) for 4 weeks during their hospitalization. Psychopathological status of the patients was assessed by senior physicians using the positive and negative syndrome scale (PANSS), consisting of positive, negative, and general psychopathology subscales.

Participants with the following characteristics were excluded from the study: unexplained first episode of SCZ; having been withdrawn from amisulpride treatment; having had amisulpride administered within the last 4 weeks; not having any follow-up blood and fecal samples; presence of infection, diarrhea, or gastrointestinal diseases; and having had antibiotics or probiotics administered within one month of recruitment. This study included 41 subjects. Five patients were excluded because they did not have follow-up blood or fecal samples, two because they had amisulpride administered less than 4 weeks ago, and one because he received another antipsychotic treatment, resulting in a final sample of 33 participants.

Participants received a standard hospital diet (i.e., 2000 ± 100 kcal, 55% ± 2% carbohydrates, 17% ± 2% protein, and 28% ± 2% fat per day), the same daily activities, psychoeducational activities, and occupational therapy. Fasting blood or fecal samples were collected following an acute episode admission on the following morning and then again the day before discharge in the morning.

2.2. Cytokine Analyses. Interleukin- (IL-) 1β, IL-4, IL-6, IL-10, tumor necrosis factor- (TNF-) α, and interferon- (IFN-) γ were assayed by BD Human Enhanced Sensitivity Cytometric Bead Array Kit (BD Biosciences, New Jersey, USA) as described previously [11].

2.3. Sampling, DNA Extraction, and 16S rRNA Gene Amplification Sequencing, Bioinformatic, and Predictive Function. All fresh fecal samples were collected and stored in a Microbiome Test Kit (G-BIO Biotech, Inc., Hangzhou, China). Total DNAs were extracted from fecal samples following the manufacturer's instructions. 16S rRNA sequencing was conducted as described for our previous study [15]. Based on the Quantitative Insights into Microbial Ecology bioinformatic pipeline for performing taxonomy assignments utilizing the operational taxonomic unit method, the total sequence data were used to analyze the fecal microbiota taxa. The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States bioinformatic software package and the Kyoto Encyclopedia of Genes and Genomes (KEGG) were utilized to predict bacterial metabolic functions.

2.4. Statistical Analyses. Values are presented as the mean ± standard deviation. We analyzed differences in gut microbiota using the Wilcoxon signed rank sum test and performed principal coordinate analysis (PCoA) on the basis of the Bray–Curtis distance function, using R software (version 3.6.0). We performed chi-square and t-test analyses using the Statistical Program for the Social Sciences version 19.0 (SPSS Inc., Chicago, IL, USA). The significance level was set at 0.05.

3. Results

3.1. Participant Characteristics. Participants included 31 males and two females. After amisulpride treatment for 4 weeks, patients had lower diastolic blood pressure ($t = 2.929$, $P < 0.005$) and heart rate ($t = 3.730$, $P < 0.001$), but increased levels of triglyceride ($t = -2.147$, $P = 0.036$; Table 1). The follow-up data showed that IL-4 levels ($t = -1.990$, $P = 0.050$) were significantly increased and IL-6 levels ($t = 2.039$, $P = 0.046$) were decreased after amisulpride treatment (Table 1).

Using the PANSS, patients with schizophrenia with acutely exacerbated symptoms receiving amisulpride

Table 1: Changes of metabolic parameters and cytokines in amisulpride-treated patients. SCZ-AE: acute exacerbated schizophrenic patients; SCZ-T: SCZ-AE treated with amisulpride for 4 weeks; NA: not analyzed.

	SCZ-AE ($n = 33$)	SCZ-T ($n = 33$)	t-test/chi-square test	P value
Age (years)	38.5 ± 11.8	—	NA	NA
Height (cm)	170.7 ± 5.6	—	NA	NA
Body weight (kg)	67.4 ± 5.1	68.0 ± 5.1	-0.486	0.629
Systolic blood pressure (mmHg)	127.5 ± 12.0	126.9 ± 8.2	0.227	0.821
Diastolic blood pressure (mmHg)	83.4 ± 7.8	78.5 ± 5.3	2.929	0.0047
Heart rate (beats per minute)	83.8 ± 9.6	76.5 ± 5.5	3.730	0.00041
Fasting blood sugar (mmol/L)	5.7 ± 2.8	5.1 ± 2.6	0.997	0.322
Triglyceride (mmol/L)	1.2 ± 0.7	1.6 ± 0.8	-2.147	0.036
Total cholesterol (mmol/L)	4.4 ± 0.8	4.6 ± 0.8	-1.094	0.278
High-density lipoprotein cholesterol (mmol/L)	1.5 ± 0.4	1.6 ± 0.5	-1.316	0.193
Low-density lipoprotein cholesterol (mmol/L)	2.6 ± 0.8	2.6 ± 0.8	0.022	0.982
Interleukin-1β (pg/mL)	8.79 ± 6.74	7.14 ± 6.93	0.979	0.331
Interleukin-4 (pg/mL)	1.87 ± 0.82	2.53 ± 1.73	-1.990	0.050
Interleukin-6 (pg/mL)	6.83 ± 2.80	5.41 ± 2.86	2.039	0.046
Interferon-γ (pg/mL)	1.59 ± 0.31	1.72 ± 0.53	-1.243	0.218
Interleukin-10 (pg/mL)	0.67 ± 0.18	0.69 ± 0.30	-0.393	0.696
Tumor necrosis factor-α (pg/mL)	2.80 ± 0.48	2.56 ± 0.62	1.758	0.084

Table 2: Clinical disability measures. SCZ-AE: acute exacerbated schizophrenic patients; SCZ-T: SCZ-AE treated with amisulpride for 4 weeks.

PANSS subscales	SCZ-AE ($n = 33$)	SCZ-T ($n = 33$)	t-test/chi-square test	P value
Positive symptoms	24.1 ± 5.2	8.3 ± 1.7	16.772	<0.001
Negative symptoms	25.5 ± 6.3	13.5 ± 3.7	9.44	<0.001
General psychopathology	48.3 ± 5.6	27.3 ± 4.7	16.475	<0.001
Total scores	96.3 ± 11.5	49.8 ± 6.4	20.316	<0.001

treatment for four weeks improved positive ($t = 16.772$, $P < 0.001$) and negative symptoms ($t = 9.440$, $P < 0.001$), general psychopathology ($t = 16.475$, $P < 0.001$), and total scores ($t = 20.316$, $P < 0.001$) (Table 2).

3.2. Characteristics of Sequencing Data. In terms of alpha diversity, we found that the difference in operational taxonomic units (OTUs) between amisulpride treatment before and after was not statistically significant (Figure 1(a)). After equalizing the library size to the minimum library size by random subtraction, we checked the average community diversity index (Chao (Figure 1(b)), Shannon (Figure 1(c)), and Simpson (Figure 1(d))) after equalizing library sizes to the minimum library size by random subtraction. We detected no statistically significant differences in community richness, diversity, and dissimilarity rank distribution (Figure 1(e)) before and after amisulpride treatment.

3.3. Alterations in Taxa Post Amisulpride Treatment. In terms of beta diversity, the gut microbiota before and after amisulpride treatment differed according to PCoA 1 and PCoA 2 (30.6% and 11.7%, respectively, Figure 2(a)). At the phylum level, we found no significant differences in relative amounts (data not shown). The relative amounts at the genus level were altered post amisulpride treatment in that *Dorea* ($P = 0.031$), *Desulfovibrio* ($P = 0.045$), and *Butyricicoccus* ($P = 0.012$) were increased, and *Actinomyces* ($P = 0.042$) and *Porphyromonas* ($P = 0.045$, Figure 2(d)) were decreased.

3.4. Predictive Functional Analysis. According to KEGG, the pathway of butanoate metabolism ($P = 0.028$) was significantly lower in the fecal microbiome post amisulpride treatment (Figure 2(e)).

4. Discussion

In this study, we found that inpatients treated with amisulpride for four weeks, showed elevated levels of IL-4 and decreased levels of IL-6. The relative amounts of genera in gut microbiome were altered. The genera of *Dorea*, *Desulfovibrio*, and *Butyricicoccus* were significantly increased; by

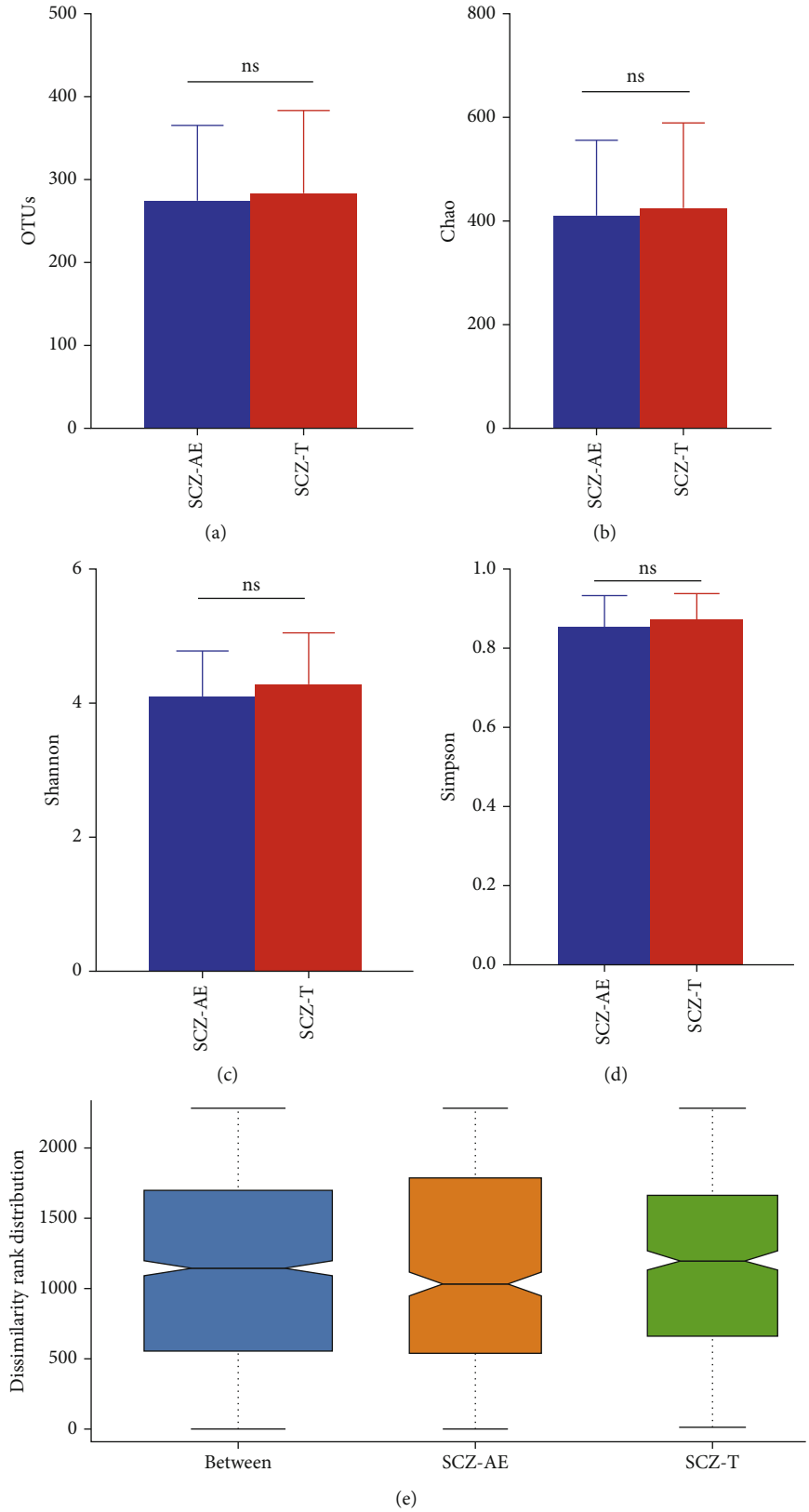
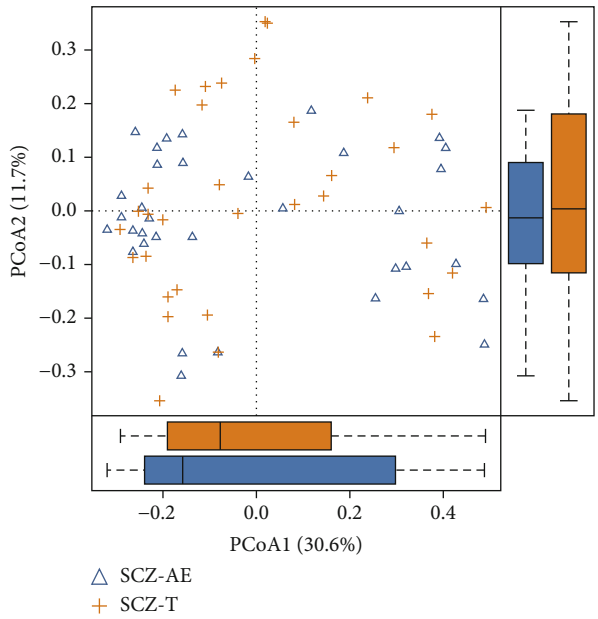
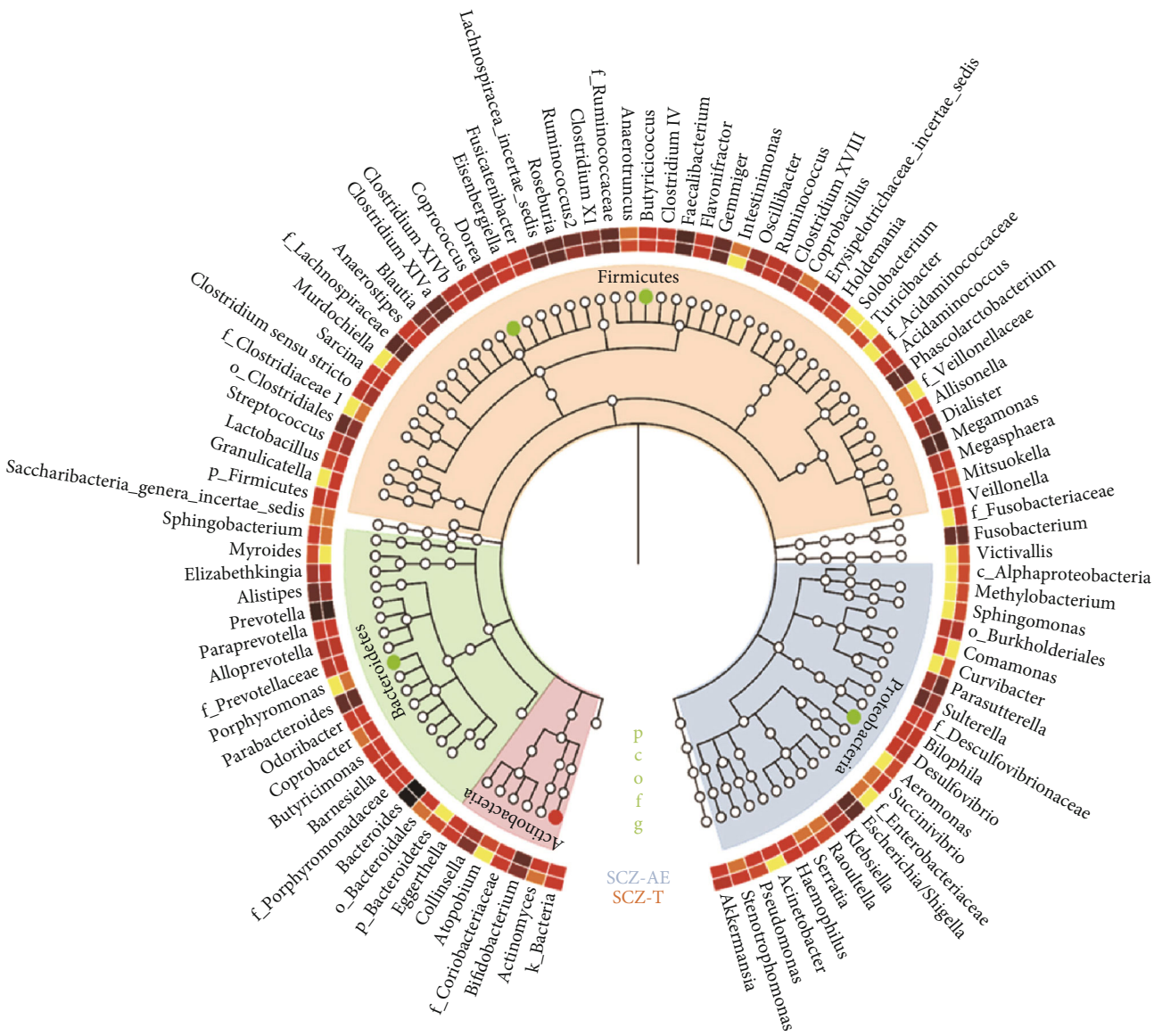


Figure 1: Summary of sequencing data. Characteristics of sequencing data in the operational taxonomic units (OTUs) (a), the mean community diversity indices (Chao (b), Shannon (c), and Simpson (d)), and dissimilarity rank distribution (e). SCZ-AE: acute exacerbated schizophrenic patients; SCZ-T: treated with amisulpride for 4 weeks; ns indicates $P > 0.05$.



(a)

Figure 2: Continued.



(b)

Figure 2: Continued.

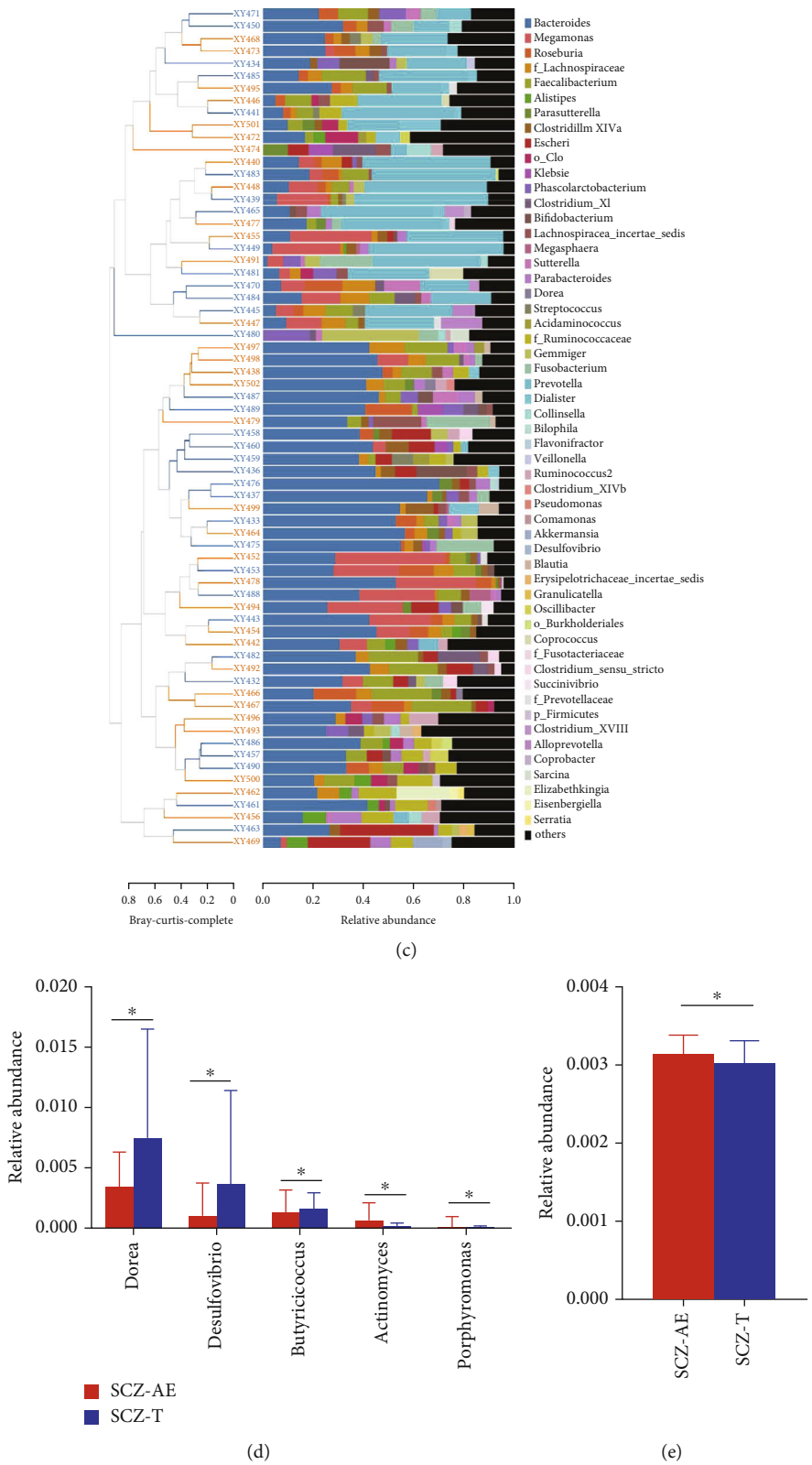


Figure 2: Gut microbiome composition at genus levels exhibits different patterns for acute patients with schizophrenia with acutely exacerbated symptoms (SCZ-AE) and those treated with amisulpride for 4 weeks (SCZ-T). Principal coordinate analysis (PCoA) based on the distance function of Bray–Curtis (a). Cladogram using LEfSe method showed the largest differences in taxa between SCZ-AE and SCZ-T (b). The color-coded bar chart shows the most prevalent genera present before and after in amisulpride treatment (c). Differences in the fecal microbiota at the genus level (d). Significant butanoate metabolism pathway downregulation was shown for the fecal microbiome in SCZ-T (e). Statistical analysis was performed with the Kruskal–Wallis test. $^{*}P < 0.05$ compared with SCZ-AE.

contrast, *Actinomyces* and *Porphyromonas* were significantly decreased. The downregulation of butanoate metabolism was examined from functional analysis of microbiota post amisulpride treatment.

SCZ is a complex neurodevelopmental disorder. Growing and converging evidence from both genetic and environmental studies points to immune and inflammatory mechanisms as contributing a substantial risk for the development of this disorder [16]. Interestingly, the prevalence of psychiatric patients with irritable bowel syndrome has been found to be over 50% [17], and the estimated SCZ comorbidity with this disease approaches 20% [18]. Moreover, 50% of patients with SCZ have gastritis, 92% colitis, and 88% enteritis in an autopsy study [19]. The importance of inflammation and the involvement of the gastrointestinal tract in SCZ have received attention. The gastrointestinal tract is the human body's largest mucosal immune defense line and has abundant microbiota. Studies demonstrate that the gut microbial ecosystem has important links to host health, including cognition and behavior [3]. Neuroscience and microbiology have converged to begin to elucidate the role of microbes in brain development and function [3]. Microbes communicate with the brain via the MGB axis, encompassing the immune system and the production of microbial metabolites, such as SCFAs, which supports the hypothesis that the microbiome is related to SCZ [20, 21].

Intestinal symbiotic microorganisms can play a role in host immune regulation, while the disruption of gut permeability is initially a cascading factor; a decrease in SCFA production results in intestinal barrier dysfunction. However, SCFAs can maintain gut integrity, promote mucus synthesis, and reduce bacterial translocation [22, 23]. SCFAs primarily form from microbial fermentation of dietary fiber, including acetate, propionate, and butyrate. Additionally, SCFAs can regulate immune cells and play a vital role to maintain the balance of the intestinal immune microenvironment, have a direct anti-inflammatory effect on the intestine, and reduce the host's intestinal inflammatory response, which in turn reduces the release of TNF-α, IL-2, and IL-6 through the histone deacetylase inhibitory pathway [22, 23]. In the present study, acutely exacerbated SCZ inpatients treated with amisulpride showed increased SCFA-producing bacteria (*Dorea* and *Butyricicoccus*) and reduced pathogenic bacteria (*Actinomyces* and *Porphyromonas*).

Antipsychotics (phenothiazines and thioxanthenes) decrease gram-positive and gram-negative bacteria activity except amisulpride, which is a benzamide [24]. However, amisulpride is pharmacologically equivalent to sulpiride, and sulpiride has beneficial effects on gastrointestinal ulcers [25]. Interestingly, amisulpride significantly decreases levels of proinflammatory cytokines and elevates levels of anti-inflammatory cytokines [26, 27]. The decreased activity of immune factors (immunoglobulins, B lymphocytes, and HLA-DR$^+$ cells) in patients undergoing SCZ treatment with amisulpride to balance the ratio of T-helper (Th)1/Th2 has been examined [28]. Moreover, amisulpride as a carrier combined with mesalazine, which is an anti-inflammatory drug, subsequently synthesizes to a new compound that could treat colonic diseases, and this new compound has an improved ability to decrease the levels of inducible nitric oxide synthase, cyclooxygenase-2, IL-1β, IL-6, and TNF-α [29]. Taken together, amisulpride has a potential anti-inflammatory effect. We speculate that amisulpride can regulate gut microbiota to achieve an anti-inflammatory effect.

Increasing SCFA-producing bacteria of acutely exacerbated SCZ inpatients is alleviated with amisulpride in this study. *Butyricicoccus* sp. was identified as a microorganism that could produce SCFAs [30]. Moreover, *Dorea formicigenerans* was enriched in SCZ; *Dorea* is part of the Lachnospiraceae family which can produce butyric acid [31]. Thus, it can be assumed to alleviate the inflammatory response and maintain intestinal epithelial function.

Nevertheless, large amounts of *Actinomyces* and *Porphyromonas* genera are observed in related inflammatory diseases. For example, large amounts of the *Actinomyces* genus have been identified in acute or remission SCZ patients [32]. By contrast, amisulpride treatment reduced these taxa in the current study. *Porphyromonas* sp. is a gram-negative and common periodontopathic bacterium, and we have reported large amounts of *Porphyromonas* spp. and elevated proinflammatory cytokines in patients with obstructive sleep apnea-associated hypertension [33]. Cooral administration of *Porphyromonas gingivalis* to intestine-induced diabetic mice has subsequently been found to lead to systemic inflammation and metabolic changes [34].

However, despite downregulation of butanoate metabolism analyzed from the microbiome, high levels of the pathogen *Desulfovibrio* were still present in this study, which is consistent with relevant literatures [4, 35]. *Desulfovibrio* is one of the mucin-degrading microbes, is a gram-negative bacterium, and is considered an opportunistic pathogen in the gut, as well as a potential lipopolysaccharide (LPS) producer [36, 37]. LPS causes mucin degradation, which disturbs the protection of gut mucosal surfaces and could potentially lead to a significant alteration in intestinal permeability, ultimately leading to bacterial translocation and intestinal leakage [38]. The increased circulating endotoxin levels then promote innate immunity events triggering low-grade systemic inflammatory processes, which may result in neurodevelopmental disorder [39]. Because butanoate metabolism of expression was decreased, we speculated a compensatory effect. In our previous study, where we investigated patients with schizophrenia with acutely exacerbated symptoms receiving antipsychotics, Th2 cytokine was markedly decreased in follow-up comparisons [11]. Another interpretation is that amisulpride has achieved the effect of reducing inflammation by increasing SCFA-producing bacteria and decreasing pathogenic bacteria, such that there was no need for excessive butyrate or other SCFAs. Another possibility is that production of reduced inflammatory metabolites from gut microbiota is not sufficient.

The main limitation of this longitudinal cohort study is the small sample size. Second, we did not compare the results between SCZ patients and healthy controls or patients receiving placebo. A prospective future study should be conducted with a larger sample size and healthy subjects to address this limitation. Third, we did not

comprehensively analyze levels of endotoxins, SCFAs in blood/stool, or other immunity factors in the blood. Fourth, gut microbiota is perturbed by many factors, especially pre-hospitalization diet/environmental exposure such as alcohol use or smoking was unable to obtain accurate information from acute exacerbated patients. Fifth, microbiome fecal transplants researches [6, 7] are necessary to support our findings, and thus, caution is warranted when interpreting the pharmacological mechanism of amisulpride in SCZ related to the gut microbiome.

5. Conclusions

The present findings indicate distinct alterations of the fecal microbiome of acutely exacerbated SCZ inpatients during four weeks of treatment with amisulpride. Changes in the microbiome are associated with increased SCFA microbiota and reduced pathogen levels, as well as regulating the balance of cytokines but downregulating butanoate metabolism. This study provides some insights in the SCZ setting into the pharmacological mechanism of amisulpride in modifying the gut microbiome.

Data Availability

All the data used to support the findings of this study are included in the article.

Ethical Approval

This study was approved by the Institutional Review Board of the Third Hospital of Quanzhou (IRB No. 2018001).

Conflicts of Interest

The authors disclose no conflicts.

Authors' Contributions

Conception and design were performed by KCY, ZJ, and LZ. Acquisition of data was performed by ZJ, WJ, and LY. Analysis and interpretation of data were performed by KCY, ZJ, and XJH. Drafting/revising of the article was performed by KCY and ZJ. Jinchi Zheng, Zeya Lin, and Chih-Yuan Ko contributed equally to this work.

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References

[1] J. Long, G. Huang, W. Liang et al., "The prevalence of schizophrenia in mainland China: evidence from epidemiological surveys," *Acta Psychiatrica Scandinavica*, vol. 130, no. 4, pp. 244–256, 2014.

[2] B. Golofast and K. Vales, "The connection between microbiome and schizophrenia," *Neuroscience and Biobehavioral Reviews*, vol. 108, pp. 712–731, 2020.

[3] J. R. Kelly, C. Minuto, J. F. Cryan, G. Clarke, and T. G. Dinan, "The role of the gut microbiome in the development of schizophrenia," *Schizophrenia Research*, vol. 234, pp. 4–23, 2021.

[4] Y. Shen, J. Xu, Z. Li et al., "Analysis of gut microbiota diversity and auxiliary diagnosis as a biomarker in patients with schizophrenia: a cross-sectional study," *Schizophrenia Research*, vol. 197, pp. 470–477, 2018.

[5] X. Zhang, L.-Y. Pan, Z. Zhang, Y.-Y. Zhou, H.-Y. Jiang, and B. Ruan, "Analysis of gut mycobiota in first-episode, drug-naive Chinese patients with schizophrenia: a pilot study," *Behavioural Brain Research*, vol. 379, p. 112374, 2020.

[6] P. Zheng, B. Zeng, M. Liu et al., "The gut microbiome from patients with schizophrenia modulates the glutamate-glutamine-GABA cycle and schizophrenia-relevant behaviors in mice," *Science Advances*, vol. 5, no. 2, p. 8317, 2019.

[7] F. Zhu, R. Guo, W. Wang et al., "Transplantation of microbiota from drug-free patients with schizophrenia causes schizophrenia-like abnormal behaviors and dysregulated kynurenine metabolism in mice," *Molecular Psychiatry*, vol. 25, no. 11, pp. 2905–2918, 2020.

[8] R. Xu, B. Wu, J. Liang et al., "Altered gut microbiota and mucosal immunity in patients with schizophrenia," *Brain, Behavior, and Immunity*, vol. 85, pp. 120–127, 2020.

[9] C.-Y. Ko and Y.-P. Liu, "Disruptions of sensorimotor gating, cytokines, glycemia, monoamines, and genes in both sexes of rats reared in social isolation can be ameliorated by oral chronic quetiapine administration," *Brain, Behavior, and Immunity*, vol. 51, pp. 119–130, 2016.

[10] M. A. Conlon and A. R. Bird, "The impact of diet and lifestyle on gut microbiota and human health," *Nutrients*, vol. 7, pp. 17–44, 2015.

[11] Y.-C. Kao, C.-Y. Ko, S.-C. Wang, and Y.-P. Liu, "Protective effects of quetiapine on metabolic and inflammatory abnormalities in schizophrenic patients during exacerbated stage," *The Chinese Journal of Physiology*, vol. 59, no. 2, pp. 69–77, 2016.

[12] J. Pełka-Wysiecka, M. Kaczmarczyk, A. Bąba-Kubiś et al., "Analysis of gut microbiota and their metabolic potential in patients with schizophrenia treated with olanzapine: results from a six-week observational prospective cohort study," *Journal of Clinical Medicine*, vol. 8, no. 10, p. 1605, 2019.

[13] Y. Liang and X. Yu, "Effectiveness of amisulpride in Chinese patients with predominantly negative symptoms of schizophrenia: a subanalysis of the ESCAPE study," *Neuropsychiatric Disease and Treatment*, vol. Volume 13, pp. 1703–1712, 2017.

[14] P. Men, Z. Yi, C. Li et al., "Comparative efficacy and safety between amisulpride and olanzapine in schizophrenia treatment and a cost analysis in China: a systematic review, meta-analysis, and cost-minimization analysis," *BMC Psychiatry*, vol. 18, no. 1, p. 286, 2018.

[15] C.-Y. Ko, Q.-Q. Liu, H.-Z. Su et al., "Gut microbiota in obstructive sleep apnea-hypopnea syndrome: disease-related

dysbiosis and metabolic comorbidities," *Clinical Science*, vol. 133, no. 7, pp. 905–917, 2019.

[16] E. G. Severance, R. H. Yolken, and W. W. Eaton, "Autoimmune diseases, gastrointestinal disorders and the microbiome in schizophrenia: more than a gut feeling," *Schizophrenia Research*, vol. 176, no. 1, pp. 23–35, 2016.

[17] W. E. Whitehead, O. Palsson, and K. R. Jones, "Systematic review of the comorbidity of irritable bowel syndrome with other disorders: what are the causes and implications?," *Gastroenterology*, vol. 122, no. 4, pp. 1140–1156, 2002.

[18] S. Gupta, P. S. Masand, D. Kaplan, A. Bhandary, and S. Hendricks, "The relationship between schizophrenia and irritable bowel syndrome (IBS)," *Schizophrenia Research*, vol. 23, no. 3, pp. 265–268, 1997.

[19] G. Hemmings, "Schizophrenia," *Lancet*, vol. 364, no. 9442, pp. 1312-1313, 2004.

[20] D. Erny, A. L. Hrabě de Angelis, D. Jaitin et al., "Host microbiota constantly control maturation and function of microglia in the CNS," *Nature Neuroscience*, vol. 18, no. 7, pp. 965–977, 2015.

[21] R. M. Stilling, M. van de Wouw, G. Clarke, C. Stanton, T. G. Dinan, and J. F. Cryan, "The neuropharmacology of butyrate: the bread and butter of the microbiota-gut- brain axis?," *Neurochemistry International*, vol. 99, pp. 110–132, 2016.

[22] M. A. R. Vinolo, H. G. Rodrigues, R. T. Nachbar, and R. Curi, "Regulation of inflammation by short chain fatty acids," *Nutrients*, vol. 3, no. 10, pp. 858–876, 2011.

[23] D. J. Morrison and T. Preston, "Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism," *Gut Microbes*, vol. 7, no. 3, pp. 189–200, 2016.

[24] H. Nehme, P. Saulnier, A. A. Ramadan et al., "Antibacterial activity of antipsychotic agents, their association with lipid nanocapsules and its impact on the properties of the nanocarriers and on antibacterial activity," *PLoS One*, vol. 13, no. 1, article e0189950, 2018.

[25] D. Kim, W. Kim, S. Jeong, D. Kim, J.-W. Yoo, and Y. Jung, "Therapeutic switching of sulpiride, an anti-psychotic and prokinetic drug, to an anti-colitic drug using colon-specific drug delivery," *Drug Delivery and Translational Research*, vol. 9, no. 1, pp. 334–343, 2019.

[26] K. S. Na and Y. K. Kim, "Monocytic, Th1 and th2 cytokine alterations in the pathophysiology of schizophrenia," *Neuropsychobiology*, vol. 56, pp. 55–63, 2007.

[27] X. He, Q. Ma, Y. Fan et al., "The role of cytokines in predicting the efficacy of acute stage treatment in patients with schizophrenia," *Neuropsychiatric Disease and Treatment*, vol. - Volume 16, pp. 191–199, 2020.

[28] T. P. Vetlugina, O. A. Lobacheva, E. N. Zhukova et al., "Clinical and experimental research of immunomodulatory effect of amisulpride," *Vestn Ross Akad meditsinskikh Nauk*, vol. 67, no. 12, pp. 13–17, 2012.

[29] W. Kim, D. Kim, S. Jeong et al., "Conjugation of amisulpride, an anti-psychotic agent, with 5-aminosalicylic acid via an azo bond yields an orally active mutual prodrug against rat colitis," *Pharmaceutics*, vol. 11, no. 11, p. 585, 2019.

[30] A. Geirnaert, M. Calatayud, C. Grootaert et al., "Butyrate-producing bacteria supplemented _in vitro_ to Crohn 's disease patient microbiota increased butyrate production and enhanced intestinal epithelial barrier integrity," *Scientific Reports*, vol. 7, no. 1, p. 11450, 2017.

[31] F. Zhu, Y. Ju, W. Wang et al., "Metagenome-wide association of gut microbiome features for schizophrenia," *Nature Communications*, vol. 11, no. 1, p. 1612, 2020.

[32] R. Pan, X. Zhang, J. Gao, W. Yi, Q. Wei, and H. Su, "Analysis of the diversity of intestinal microbiome and its potential value as a biomarker in patients with schizophrenia: a cohort study," *Psychiatry Research*, vol. 291, p. 113260, 2020.

[33] C. Y. Ko, A. K. Hu, D. Chou et al., "Analysis of oral microbiota in patients with obstructive sleep apnea- associated hypertension," *Hypertension Research*, vol. 42, no. 11, pp. 1692–1700, 2019.

[34] A. Ohtsu, Y. Takeuchi, S. Katagiri et al., "Influence of Porphyromonas gingivalis in gut microbiota of streptozotocin-induced diabetic mice," *Oral Diseases*, vol. 25, no. 3, pp. 868–880, 2019.

[35] V. L. Nikolova, M. R. B. Hall, L. J. Hall, A. J. Cleare, J. M. Stone, and A. H. Young, "Perturbations in gut microbiota composition in psychiatric disorders: a review and meta-analysis," *JAMA Psychiatry.*, vol. 78, no. 12, pp. 1343–1354, 2021.

[36] S. Xiao, N. Fei, X. Pang et al., "A gut microbiota-targeted dietary intervention for amelioration of chronic inflammation underlying metabolic syndrome," *FEMS Microbiology Ecology*, vol. 87, no. 2, pp. 357–367, 2014.

[37] T. Shao, L. Shao, H. Li, Z. Xie, Z. He, and C. Wen, "Combined signature of the fecal microbiome and metabolome in patients with gout," *Frontiers in Microbiology*, vol. 8, p. 268, 2017.

[38] S. S. Ghosh, J. Wang, P. J. Yannie, and S. Ghosh, "Intestinal barrier dysfunction, LPS translocation, and disease development," *Journal of the Endocrine Society*, vol. 4, no. 2, 2020.

[39] M. Escobar, N. Crouzin, M. Cavalier et al., "Early, time-dependent disturbances of hippocampal synaptic transmission and plasticity after in utero immune challenge," *Biological Psychiatry*, vol. 70, no. 10, pp. 992–999, 2011.

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Research Article

Neurobehavioral Differences of Valproate and Risperidone on MK-801 Inducing Acute Hyperlocomotion in Mice

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Objective. The glutamate system plays a major role in the development of neuropsychiatric disorders such as addiction, epilepsy, dementia, and psychosis. MK-801 (dizocilpine), an uncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, could increase locomotor activity and stereotyped neurobehaviors mimicking schizophrenic-like features in the mouse model. The study would explore the neuropharmacological differences of risperidone and valproic acid on the MK-801-induced neurobehavioral changes. *Methods*. The subjects were male C57BL/6J mice obtained from the National Laboratory Animal Center. Drug effects were assessed using the open field with a video-tracking system and gaiting tests. After habitation, risperidone (0, 0.1 mg/kg) or valproic acid (0, 200 mg/kg) was injected and ran locomotion for 30 mins. Sequentially, mice were followed by intraperitoneal injection (i.p.) with MK-801 (0, 0.2 mg/kg) and ran locomotion for 60 mins. Gaiting behaviors such as step angles, stride lengths, and stance widths were measured following the study drugs. *Results*. The results showed that risperidone and valproic acid alone could not alter the locomotor activities. Following the MK-801 injection, the travelled distance and speed in the entire open field dramatically increased. The dose 0.1 mg/kg of risperidone could totally inhibit the MK-801-induced hyperlocomotion compared with that of the saline-injected group ($p < 0.001$). The valproic acid (200 mg/kg) partially suppressed the hyperlocomotion which is induced by MK801. *Conclusion*. The more dominant effect of risperidone to rescue MK-801 induced hyperlocomotion compared with that of valproic acid. The partial suppression of valproic acid may imply the psychopharmacological evidence as adjuvant effect to treat psychotic patients through tuning glutamatergic neurotransmission.

1. Introduction

Under the clinical circumstance, the agitation, aggression, and psychosis are devasting symptoms in major psychiatric disorders including schizophrenia, bipolar affective disorder, substance intoxication, dementia, and epilepsy [1]. Such disruptive symptoms can significantly raise the healthcare burden including the emergency department, hospitalization, and outpatient setting and even in the community [2]. Although there are numerous neuroscience researches to explore the basic mechanisms of psychotic disorders, to date, there is a lack of convincing reports in the understanding of neurobiological mechanisms underlying psychosis. Growing evidence demonstrates that the glutamate system plays a major role in the development of addiction, schizophrenia, dementia, and other mental disorders [3–6].

Risperidone, an atypical antipsychotic, is widely used to control the acute psychosis through reducing hyperdopaminergic neurotransmission. It is not only approved to treat schizophrenic disorder but also considered as a mood

stabilizer in various bipolar treatment guidelines including the World Federation of Societies of Biological Psychiatry (WFSBP) [7, 8], National Institute for Health and Care Excellence (NICE) [9], and Canadian Network for Mood and Anxiety Treatments (CANMAT) [10]. Valproic acid, an anticonvulsant, is used as a mood stabilizer and is proved in the above treatment guidelines for bipolar affective disorders. Treatment guidelines also highlighted that the augmentations of valproic acid and atypical antipsychotics, such as risperidone, have favorable treatment effect over bipolar patients who failed to respond to monotherapy.

In the past two decades, the interactions between psychosis and the glutamate system have been proposed by the *N*-methyl-D-aspartate (NMDA) receptor hypofunction, as the administration of NMDA antagonists has been observed to contribute to psychotomimetic actions other than sedation, anesthetic, and antidepressant effects [11, 12]. Abusers of NMDA receptor antagonists, such as ketamine and phencyclidine (PCP), would experience agitation, perceptual distortions, hallucinations, and delusions resembling positive symptoms of schizophrenia [4, 6, 13]. The addictive properties of these psychoactive substances are related to their tuning the dopaminergic and glutamatergic neurotransmission in certain brain areas such as the prefrontal cortex, ventral tegmental area, and nucleus accumbens, deriving from the NMDA receptor blockade [14]. It will be a curious issue to find a treatment module attenuating the agitated and psychotic symptoms in the subjects with psychoactive addiction.

MK-801 (dizocipline), an uncompetitive NMDA receptor antagonist, has been proven to induce acute excitation, agitation, or aggressive behaviors in different animal models [5, 15, 16]. Memory impairment and learning impairment were also seen in the MK-801 animal model, resembling negative symptoms of schizophrenia through modulation of Rho family protein-related signaling [17]. MK-801-induced neurobehavioral alteration was adapted as an acute schizophrenic-like model in animal studies [15, 18]. Since antipsychotics act to reduce the hyperdopaminergic neurotransmission, the interactions between dopamine and glutamate neuron may play an important role in the development of psychosis from the treatment modality. In animal model, the MK-801-induced hyperlocomotion could be reduced by antipsychotic administration such as risperidone [19] and the results were compatible to clinical findings of risperidone treatment of active psychotic patients. There was a report that valproic acid could prevent the induction of MK-801 sensitization and block the behavioral cross-sensitization of methamphetamine and MK-801 [20]. Clinical evidences have showed the effectiveness of adjunctive therapy by valproic acid and antipsychotics for treating schizophrenia with symptoms of agitation, aggression, and excitation [21–25]. Valproic acid has also been adapted in treatment guidelines for the treatment of aggression and hostility symptoms in schizophrenia [26]. However, the neurobiological mechanism of the valproic acid effect on psychosis remains unknown [27]. Currently, the psychiatric diagnostic systems could not deduce the agitation and disorganized behaviors into one singular diagnosis. Such behavioral manifestations could be a part of diverse psychiatric disorders, including schizophrenia, affective disorders, substance intoxication, and withdrawal state [28–30].

Until now, there is a lack of neurobiological basis to support the pharmacodynamic effect of valproic acid to control the agitated/aggressive features in psychotic patients. To test the valproic acid for potential interactions with glutamatergic and dopaminergic neurotransmission, the MK-801-induced hyperlocomotion was used as a positive control and compared to the risperidone pharmacological reaction. The valproic acid and risperidone were administrated alongside or in combination with MK-801. The study measured the neurobehavioral changes following the separated drug injection and assessed the suppression effect on MK-801. In addition, we also compared the differences of locomotor behaviors and gaiting parameters following valproic acid and risperidone treatment.

2. Methods

2.1. Animals and Drug Preparations. Subjects were adult male C57BL/6J mice obtained from the National Laboratory Animal Center. Mice were housed as four in a cage in a temperature ($22 \pm 1^{\circ}$C) and humidity ($50 \pm 5\%$) well-controlled vivarium, under a 12-hour light/dark cycle with ad libitum access to food and water. MK-801 and valproic acid sodium salt (Sigma-Aldrich Co.) were dissolved in saline. The risperidone (Sigma-Aldrich Co.) was dissolved in 2% dimethyl sulfoxide (DMSO) and diluted in saline. The drugs were diluted for the subjects to receive 0.2 mg/kg of MK-801, 200 mg/kg of valproic acid, and 0.1 mg/kg of risperidone as injection volume by 10 mL/kg. All experimental procedures were approved by the Institutional Animal Care and Use Committee, Buddhist Taipei General Hospital, and were conducted following the regulation of reduction and refinement. All efforts were made to minimize the number of animals used and their suffering.

2.2. Open Field Test. After adaptation to laboratory and housing conditions for 60 minutes, subjects were placed in the open field for a 30-minute habituation. The open field apparatus was a square-shaped opaque device with a length and width of 40.5 cm and 35 cm in height made with acrylic material. It featured a square-shaped central region measuring 28 cm in length and width with central luminous being set at 50 ± 5 LUX. After the habituation period, subjects were randomly divided into three groups, receiving risperidone 0.1 mg/kg, valproic acid 200 mg/kg, and vehicle, through intraperitoneal injection (i.p.). The mice were immediately placed in the open field for locomotion measurement for 30 minutes. Sequentially, MK-801 (0.2 mg/kg) or saline was injected and mice were placed in the open field testing for 60 minutes; the experimental design is demonstrated in Supplement 1. The locomotor activities were recorded via a video-tracking system (SINGA, TW), as shown in Supplement 2 (marked as blue lines). The general experiment procedures were well conducted by negative and positive controls, saline and MK-801, respectively. The drug testing

study was separately performed and combined to compare the difference in the same module.

2.3. Gaiting Test. The gaiting test was conducted to evaluate the behavioral quality of the mouse behavior. [31]. The gait recording apparatus was a rectangle-shaped device measuring 5 cm in width and 80 cm in length. The luminous was set at 120 ± 10 LUX from the starting point of the apparatus, and 70 ± 10 LUX at the end of the apparatus. The environmental luminous was set at 15 ± 5 LUX. After adaptation to laboratory and housing conditions, subjects were randomly divided into three groups, receiving risperidone 0.1 mg/kg, valproic acid 200 mg/kg, and vehicle, through intraperitoneal injection (i.p.). Subjects were then wetted by ink on hind paws and placed on the gait recording apparatus with strip of papers to record the gaits as the subjects proceed from the starting point to the end. After the first injection, gaits were recorded at 0, 10, 20, and 30 minutes postinjection. Sequentially, mice were followed by a second intraperitoneal injection with MK-801 (0.2 mg/kg) or saline and gaits were assessed at 0, 10, 20, 30, 40, 50, and 60 minutes postinjection. Parameters of the gait were then measured and recorded including the step angles, stride lengths, and stance widths. In the gaiting test, the three consecutive longest footprints were used to measure each parameter. Step angles were measured by the connected lines of the outer border of the hind paws. Stride lengths were measured by the distance between the front edge of the hind paws. Stance widths were measured by the vertical distance between one outer edge of the hind paw and the connected line of the stride length on the opposite side. The mean value was used to represent each parameter.

2.4. Statistical Analysis. All the statistical analyses were conducted by GraphPad Prism (GraphPad Software Inc., San Diego, CA) version 5.01.336 and Statistical Package for Social Science (SPSS Inc., Chicago, Illinois). Paired t-test and analysis of variance (ANOVA) were performed to analyze the differences of measurement parameters of the mice locomotor activities. Post hoc analysis by Scheffe's multiple comparison test was used in this study.

3. Results

3.1. The MK-801 Effect. In the open field test, there were no significant differences in the travelled distance, travelled speed, frequency of entering central area, and duration in the central area between all groups in the habituation period. The mice displayed avoidant behaviors of travelling to the central area compatible with the lower percentage of central duration and central travelled distance. In the open field test, after MK-801 injection, the vehicle + MK-801 group showed a dramatical increase in the total travelled distance and travelled speed in the entire open field. The central travelled speed, frequency of entering the central area, and central travelled distance were significantly increased ($p < 0.001$). (shown in Figures 1–3).

3.2. The Risperidone/Valproic Acid Effect. The valproic acid or risperidone alone did not affect the travelled distance. Following the 1st i.p. injection (risperidone or valproic acid), there was no significant difference in the travelled speed, frequency of entering the central area, and duration in the central area between all groups 30 minutes after the 1st i.p. injection in the open field study (see Figures 1 and 2).

3.3. Risperidone + MK-801. The results showed that the dose 0.1 mg/kg of risperidone could totally inhibit the MK-801-induced hyperlocomotion compared to that of the vehicle + MK-801 group ($F = 17.18$, $p < 0.001$) (see Figure 1).

For further imaging analysis for risperidone groups, see Figure (a) of Supplements 3–6.

3.4. Valproic Acid + MK-801. Valproic acid (200 mg/kg) attenuated the MK-801-induced hyperlocomotion. The travelled behavioral patterns showed the persistent partial suppression on the MK-801 effect at various corresponding time points (see Figure 2). However, the overall level of suppression is less significant comparing with that of risperidone.

For further imaging analysis of the valproic acid group, see Figure (b) of Supplements 3–6.

3.5. Differences of Risperidone/Valproic Acid on MK-801 Effects. There are the significant differences in the total travelled distance between vehicle + MK-801 (255 ± 51 meters) and risperidone + MK-801 (43 ± 3 meters) ($p < 0.001$); while valproic acid showed partial suppression on MK-801-induced hyperlocomotion, the total travelled distances were 257 ± 39 and 194 ± 12 meters in vehicle + MK-801 and valproic acid + MK-801 ($p = 0.2706$), respectively (see Table 1).

The significant increase of the central travelled distance, speed, and duration was noted following the MK-801 injection ($p < 0.001$). There were no significant differences between the vehicle + vehicle group, risperidone + vehicle group, and risperidone + MK-801 groups in the total travelled distance and speed, central travelled distance and speed, duration in central area, and frequency of entering the central area (see Figure 3(c)). The differences in the central travelled distance, central travelled speed, and frequency of entering the central area were seen between the MK-801-injected groups and those that did not receive MK-801, in which the groups receiving MK-801 (vehicle + MK-801 and valproic acid + MK-801) were significantly higher ($F = 15.45$, $p < 0.01$ central travelled distance; $F = 17.97$, $p < 0.001$ in frequency of entering the central area) (see Figure 3(b)). Significantly higher duration in the central area was found between the valproic acid + MK-801 group and those without MK-801 ($F = 8.51$, $p < 0.01$) (see Figure 3(d)).

3.6. Gaiting Analysis. In the gaiting test, the stride length was significantly increased after MK-801 injection ($p < 0.05$ 40 minutes after MK-801 injection and $p < 0.01$ 50 and 60 minutes after MK-801 injection) and there is no difference in other parameters regarding the step angle and stance width (see Figure 4(a)). The valproic acid or risperidone alone did not affect the gaiting behaviors including the analyses of the step angle, stride length, and stand width.

The increased stride length of mice after MK-801 injection could be reduced by prior the administration of dose 0.1 mg/

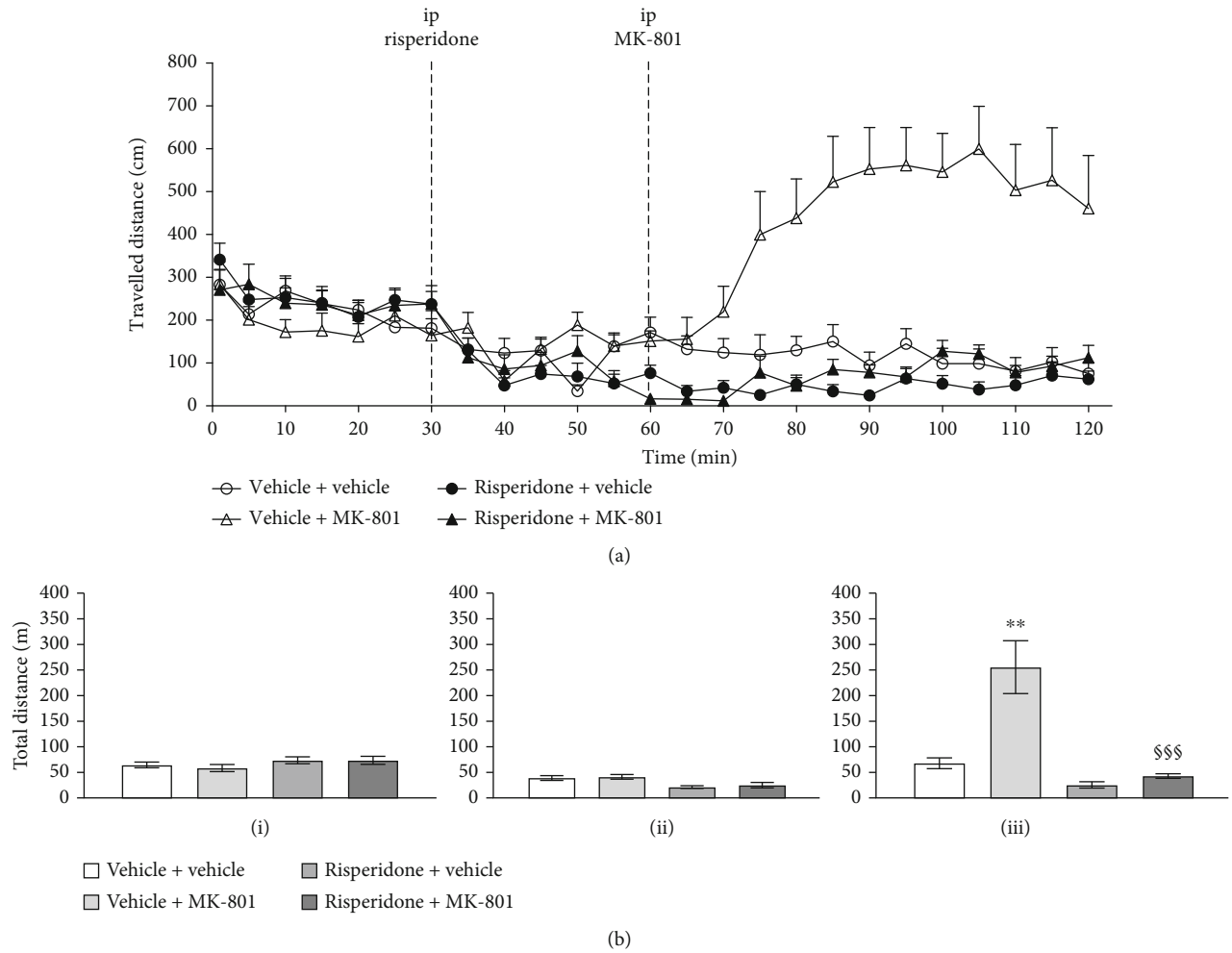


Figure 1: The travelled distance (cm) at the corresponding time points in the open field test following the risperidone and MK-801 injection (a). Comparisons of the total travelled distance (m) for each drug treatment group (b); vertical bars represent SEMs: (i) represents baseline within 0–30 minutes, (ii) represents risperidone drug effect during 30–60 minutes, and (iii) represents MK-801-injected travelling change during 60–120 minutes; total travelled distances are shown in meters. Subject numbers were 9–10 in each group. Differences among the study groups were evaluated by ANOVA and post hoc analysis. Statistically significant differences between groups: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. vehicle + vehicle; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, and $^{\S\S\S}p < 0.001$ vs. vehicle + MK-801.

kg of risperidone, and there were no significant differences between risperidone + MK-801, risperidone + vehicle, and vehicle + vehicle groups regarding stride length (see Figure 4(b)). The gaiting test showed that valproic acid could not alter the gaiting of MK-801-injected subjects. As seen in the risperidone groups, there is also an increase of stride length after MK-801 injection. However, there were no differences in the step angle and stance width between all groups receiving MK-801 (see Figure 4(c)) and both the vehicle + MK-801 group and the valproic acid + MK-801 group showed a significant increase in stride length comparing with the control group ($p < 0.01$ 40 minutes after MK-801 injection, $p < 0.001$ 50 minutes after MK-801 injection, and $p < 0.05$ 60 minutes after MK-801 injection) (see Figure 4(d)).

4. Discussion

In this study, the homogeneity of the mice strain, age, sex, and environmental factors, such as illumination, temperature, and humidity is under well control, providing a good platform to assay the neuropharmacological effects of valproic acid and risperidone following MK-801, a specifically NMDA receptor antagonist. The principal findings of this study have shown the following: (1) MK-801 persistently produces hyperlocomotion, (2) locomotor behaviors are unaffected by risperidone and valproic acid injection alone, (3) risperidone markedly suppresses the MK-801-induced hyperlocomotion, and (4) valproic acid partially inhibits the MK-801 pharmacological effects. To our knowledge, this is the first report to explore the pharmacological differences of risperidone and valproic acid in a same valid module, as comparing MK-801 modulation on the blockade of glutamatergic neurotransmission.

4.1. Drug Effects on Locomotor Activities. In this study, 0.2 mg/kg of MK-801 was employed because <0.1 mg/kg of dose may be insufficient enough to induce hyperlocomotor activity and > 0.5 mg/kg of dose may induce ataxia in male

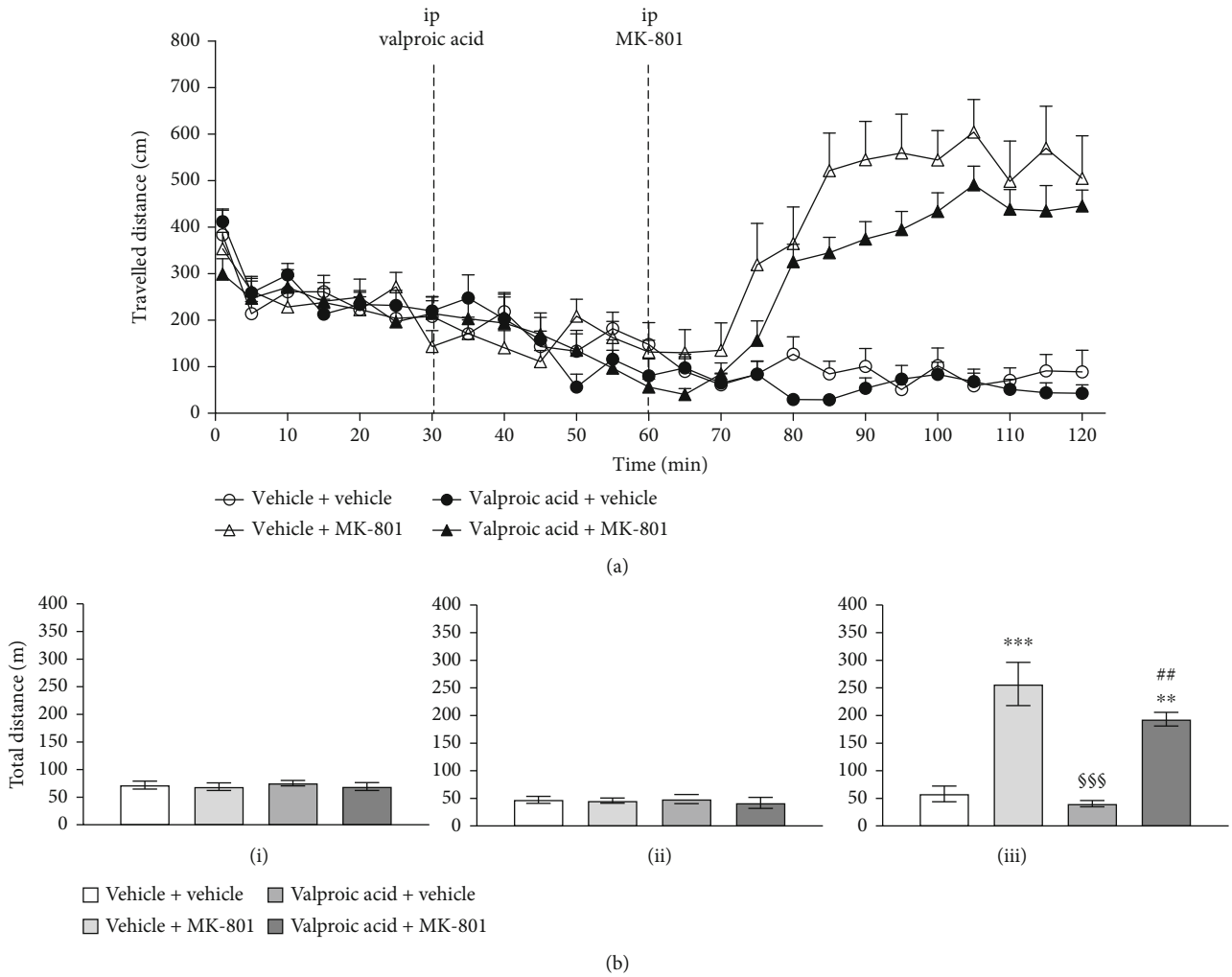
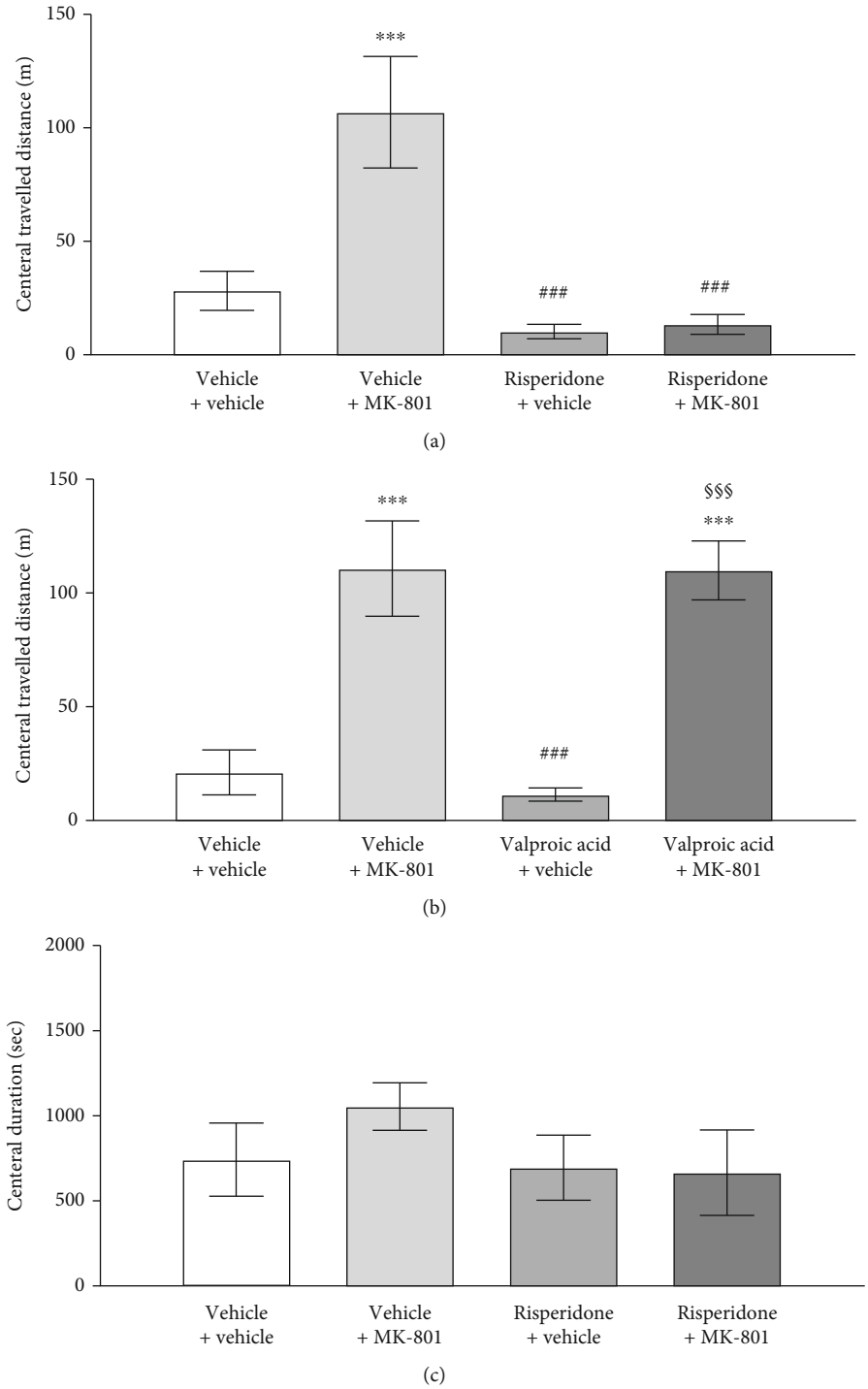


Figure 2: The travelled distance (cm) at the corresponding time points in the open field test following the valproic acid and MK-801 injection (a). Comparisons of the total travelled distance (m) for each drug treatment group (b); vertical bars represent SEMs: (i) represents baseline within 0–30 minutes, (ii) represents valproic acid drug effect during 30–60 minutes, and (iii) represents MK-801-injected travelling change during 60–120 minutes; total travelled distances are shown in meters. Subject numbers were 10–11 in each group. Differences among the study groups were evaluated by ANOVA and post hoc analysis. Statistically significant differences between groups: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. vehicle + vehicle; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ vs. valproic acid + vehicle; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, and $^{\S\S\S}p < 0.001$ vs. vehicle + MK-801.

mice which could intervene with this study [32, 33]. Doses of 0.1 mg/kg of risperidone and 200 mg/kg of valproic acid were employed based on previous related studies [34–38]. During the open field test, the MK-801-injected subjects displayed hyperlocomotion, increased frequency of entering the central area, increased total and central speed, and increased central distance, indicating the psychotomimetic effect of MK-801 resulting in overall agitation and disorganized behaviors in test subjects. The study result is consistent with the open field test of the schizophrenia glutamate model [6, 39], proposing the excitatory schizophrenia symptoms which resulted from NMDA inhibition. In this study, the hyperlocomotion could be completely suppressed by prior risperidone injection (0.1 mg/kg), implying that antagonizing of dopamine receptors, especially dopamine receptor D2, and serotonin (5-hydroxytryptamine; 5-HT) receptors, especially the 5-HT_{2A} receptor, which are main targets of atypical antipsychotics, plays a major role in inhibiting the excitation and agitated behaviors. The previous study demonstrated that antagonizing of D1 and D2 receptors, as well as 5-HT_{2A}, is necessary for the suppression of MK-801-induced hyperlocomotion [40]. Similarly, there are evidences suggesting that the psychotomimetic effects illustrated in the hyperlocomotor activity of MK-801 along with other NMDA antagonists are believed to result from the increase of the dopamine level in the prefrontal cortex [5, 41]. It is noteworthy that the agitated and excited behaviors seen in various neuropsychiatric diagnoses may be antecedent from the dopamine hyperactivation and there are also evidences that such increase in dopamine neuron firing is regulated by NMDA-mediated glutamatergic neurons [5, 41]. The positive psychotomimetic effects of ketamine and PCP are believed to be derived from the inhibition of the NMDA receptor on the γ-aminobutyric acid (GABA)



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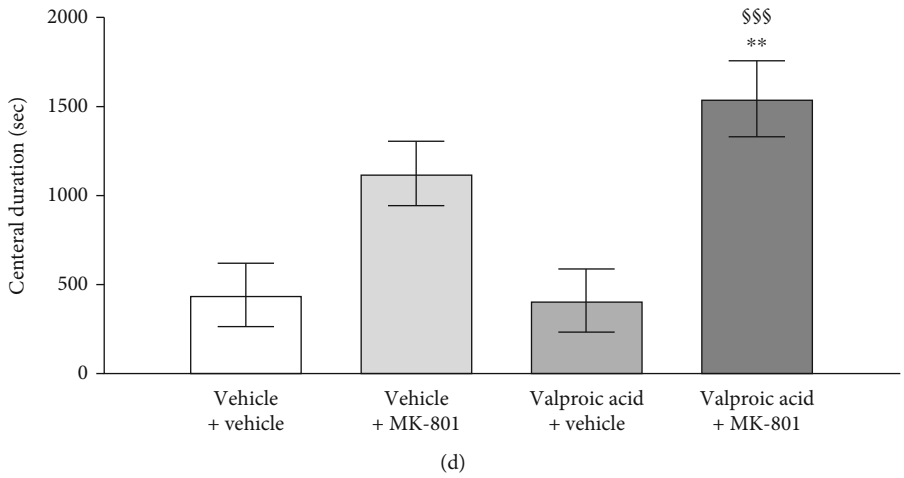


Figure 3: Alterations of the central travelled distance (a, b) and central duration (c, d) followed by MK-801 injection in the open field for mice treated with risperidone/valproic acid. Central travelled distances and duration are shown in meters and seconds, respectively. Vertical bars (for clarity, only the upper or lower portions shown) represent standard errors of the means. Statistically significant differences between groups were evaluated by ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. vehicle + vehicle; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ comparing with vehicle + MK-801; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, and $^{\S\S\S}p < 0.001$ vs. valproic acid + vehicle.

Table 1: Comparisons of risperidone and valproic acid effects on MK-801-induced hyperlocomotion.

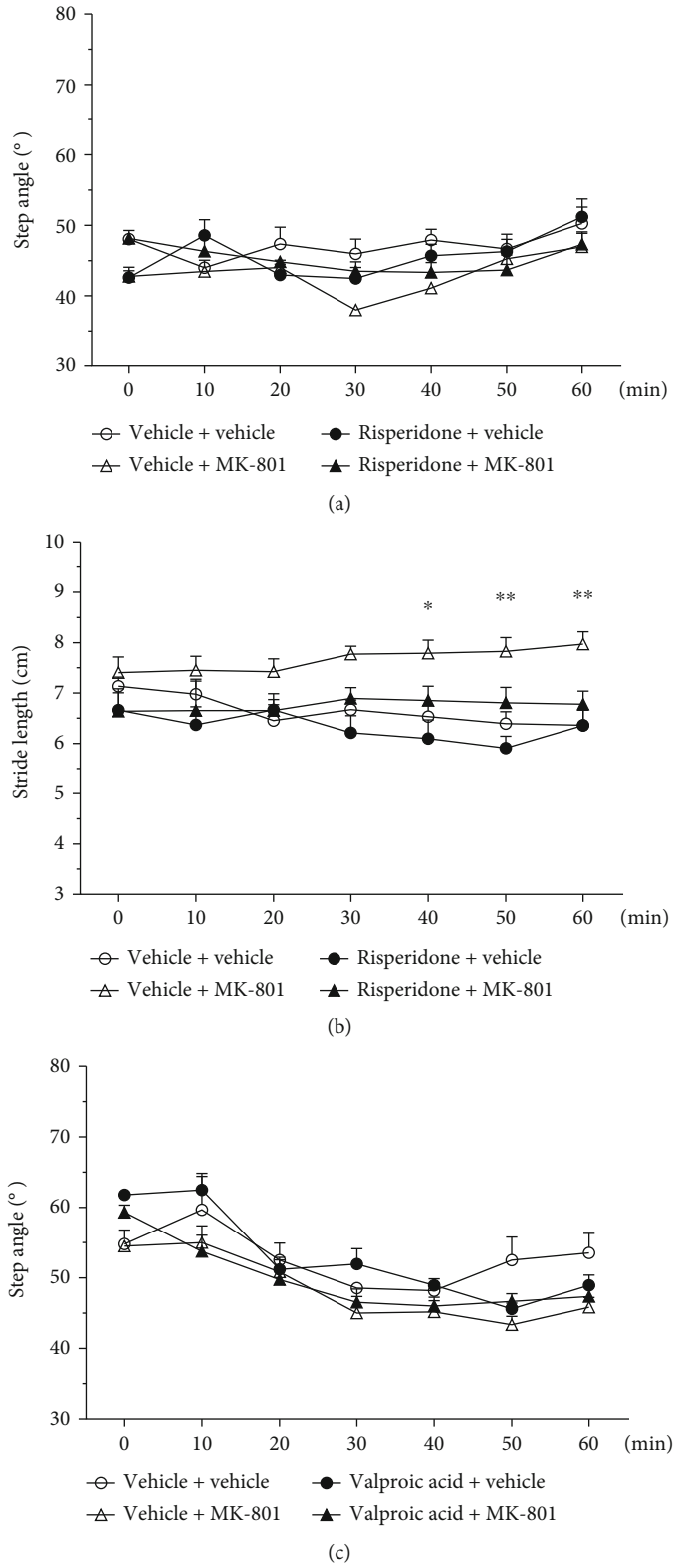
MK-801 dose (mg/kg)	Risperidone dose		Valproic acid dose	
	0 mg/kg	0.1 mg/kg	0 mg/kg	200 mg/kg
0	67.2 ± 10.3	25.2 ± 6.0	58.0 ± 14.4	40.4 ± 5.6
0.2	255 ± 51.2**	42.5 ± 4.3†††	257 ± 39.2***	194 ± 12.3**,§§

Values are means ± SEM. Data represent the total travelled distance (meter) in 60 minutes following MK-801 injection. Differences among the study group were evaluated by multiple analysis of variance and post hoc analysis. Subject numbers were 9–11 in each group. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. the 0 mg/kg MK-801 group; $^{\dagger\dagger\dagger}p < 0.01$ vs. the 0 mg/kg risperidone group; $^{\S\S}p < 0.01$ vs. the 200 mg/kg valproic acid + 0 mg/kg MK-801 group.

neuron, resulting in hyperdopaminergic transmission [42, 43]. Being a structural analog of PCP and ketamine [44], MK-801 acts on the central glutamate NMDA receptor, inhibits the glutamatergic transmission, may lead to decreased GABAergic neuron firing, and disinhibits dopamine neurotransmission [45].

4.2. Drug Effects on the Gaiting Test. Other than hyperlocomotor activity, the acute MK-801 administration can also result in a specific motor pattern of ataxia and stereotypy in injected mice which may interfere with the open field performances [15], and in this study, the gaiting test was performed to evaluate the behavioral quality of the mice after receiving testing drugs. The significant differences in stride length of the vehicle + MK-801 group vs. vehicle + vehicle group indicated the behavioral change in MK-801 injection, as the tested mice tended to take longer steps after injection. The MK-801 induced longer stride lengths and was rescued by risperidone treatment, as shown in Figures 3 and 4 of Supplement 7. The result is compatible to the findings in the open field test as reflected in the increased total travelled distance and travelling speed [31]. It may indicate that the increased stride length after MK-801 injection is a phenomenon of excitation/agitation rather than drug adverse effects such as ataxia or stereotypy. The MK-801 + valproic acid group showed no such reduction in stride length, suggesting an inferior ability of valproic acid comparing to risperidone in glutamatergic suppression-induced excitation/agitation, as shown in Figures 3 and 4 of Supplement 8 (see Figure 4(d)). In addition, no differences were observed between the risperidone + vehicle group and vehicle + vehicle group regarding all gaiting parameters including the step angle, stride length, and stance width, suggesting that the antipsychotic suppression of the hyperlocomotion seen in the open field test is not a result of the drug effect over the subject's movement quality, but a suppression of hyperlocomotion.

4.3. Interplay of Neurobehaviors and Pharmacodynamic Effects between Drugs. In this study, the valproic acid-(200 mg/kg) injected subjects showed partial suppression over the hyperlocomotion induced by MK-801, implying an adjuvant effect over the excitation and agitation behaviors of acute neuropsychiatric symptoms. It is not uncommon that valproic acid is utilized in treating patients with agitation/excitation symptoms; while valproic acid monotherapy for manic episode in bipolar affective disorder patients is frequently used with good clinical evidence, it is rarely used as monotherapy in schizophrenia or substance intoxication/withdrawal patients. It may raise questions that whether the agitated and excitation behaviors presented in each disorder have a common mechanism and could be all managed by valproic acid. There have been preliminary investigations of valproic acid use in schizophrenia patients [27, 46],



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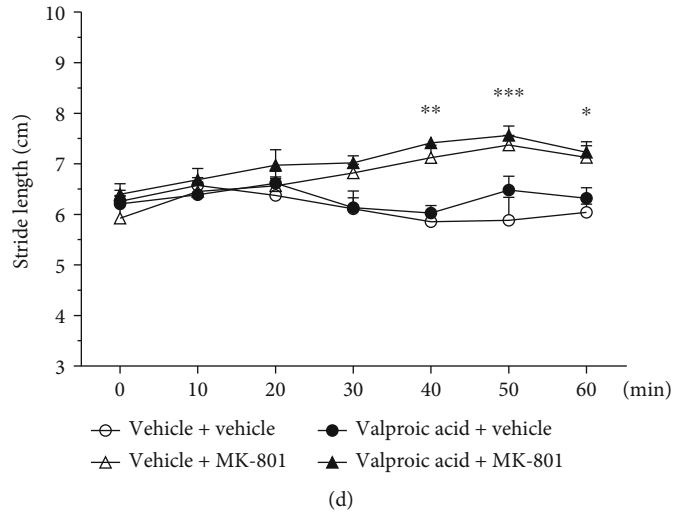

Figure 4: Behavioral differences of gaiting, step angle (a, c), and stride length (b, d) followed by MK-801 injection for mice treated with risperidone/valproic acid. Statistically significant differences between groups: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. vehicle + vehicle. Vertical bars represent SEMs.

especially in violent and aggressive patients, but with mixed results. Up to this date, valproic acid monotherapy is not approved for the treatment of schizophrenia; in most articles and treatment guidelines, valproic acid remains as augmentation treatment strategy but showed being ineffective in the treatment of schizophrenia core symptoms such as hallucinations or delusions [21–24, 47]. Since the first marketing in the 1960's, valproic acid is initially used for the treatment of epilepsy and later discovered to be effective in various excitability disturbances in both central and peripheral nervous system disorders, including neuropathic pain, migraine, bipolar disorder, essential tremor, and even schizophrenia [48–50].

Valproic acid is suggested to possess multiple mechanisms of action, the major actions including the potentiation of GABAergic neuron, the blockade of voltage-dependent sodium channels, the blockade of T-type calcium channels, and the blockade and modulation of glutamatergic excitation neurons [48–50]. As mentioned above, these mechanisms of action could explain its overall inhibitory effects over neural systems of valproic acid and why it is favored in the use to control symptoms believed to cause neuron excitation, such as epilepsy. However, as demonstrated in this study, valproic acid only possesses modest effect over MK-801 or NMDA-mediated glutamate excitation behaviors comparing with risperidone. Besides the total travelled distance, the valproic acid + MK-801 group showed no difference versus the vehicle + MK-801 group in the central travelled distance, central speed, and frequency of the central entry in the open field test, in contrary to the risperidone + MK-801 group, which revealed suppression in the above parameters comparing with the vehicle + MK-801 group. In addition to open field test findings, the gaiting test also confirmed that valproic acid could not reverse the abnormal behavioral pattern induced by MK-801. This study result is compatible with the current treatment strategy for schizophrenia. Risperidone revealed a more dominant effect than valproic acid to rescue MK-801 induced hyperlocomotion, which is consistent with the clinical therapeutic effect of risperidone on acute psychosis. It may imply that the agitated/impulsive behaviors shown in schizophrenia indeed have a distinct disease entity other than what is seen in bipolar affective disorder but has some degree of overlapping which could be partially managed by adjunctive of valproic acid but could not be treated by valproic acid alone [25].

The study indicated that the partial suppression effect of valproic acid on MK-801-induced hyperlocomotion may have some similarities in these psychiatric diagnoses regarding the drug effect and neurological mechanism. Our results demonstrate MK-801 augmentation of locomotor activity via NMDA receptor blockade [6]. It might imply that the compound could attenuate the GABA function and caused the overactivity of dopaminergic neurotransmission. The dominant inhibitory effect of risperidone on MK-801 is via the inhibition of dopamine transmission. The neurobehavioral alterations of MK-801 and risperidone would raise the potential interactions of pharmacological properties between glutamate and dopamine neurotransmissions. Apart from the glutamate-dopamine crosstalk, valproic acid has been used as a psychotropic agent through balancing the GABA and glutamate neurofunction. It may provide neuropharmacological evidence for valproic acid in adjuvant to risperidone to reduce psychosis and also encourages further examining of the nature of the agitation/excitement phenotype of psychiatric patients and to consider a different treatment approach strategy.

The study adopted the open field to evaluate locomotor activity and gaiting to assess the behavioral quality, in which the drug suppression effect of MK-801 induced hyperlocomotion mimicking the treatment response of acute psychosis in the animal model. Apart from the acute excitation, the

current study module could not totally encompass the diverse symptom domain in acute psychotic subjects clinically; therefore, further study is required to explore the other symptom domains such as fearfulness, anxiety, cognitive, and negative symptoms. There are other animal models for psychotic symptoms, including prepulse inhibition (PPI), latent inhibition, social interaction and recognition, forced swim test, and other models for cognitive symptoms [5]. It can be insightful to integrate other animal models in this study module. Besides the study management, it will be an interesting issue to compare the drug reactions in other mouse strains with different genetic backgrounds. In addition, this study mostly adopted adult mice for test subjects and evidence indicated that age differences of mice test subjects will be a confounding factor to the drug sensitivity and susceptibility, especially in adolescence mice [51, 52]. Further studies also aim to integrate more sample size and comprehensive animal neurobehavioral models to clarify the valproic acid on the treatment effect of psychosis. Finally, this study examined the effect of antipsychotic and valproic acid over MK-801-induced hyperlocomotion alone but the augmentation treatment strategy with antipsychotics plus valproic acid for the acute excitation subjects was not tested and is left for future investigation, especially the interactions of dopamine, GABA, and glutamate neurotransmission.

5. Conclusion

The more dominant effect of risperidone to rescue MK-801 induced hyperlocomotion compared with valproic acid. The partial suppression of valproic acid may imply the psychopharmacological evidence as adjuvant effect to treat psychotic patients through tuning glutamatergic neurotransmission. It would be an important issue to deeply investigate our current findings through integrating the neurophysiology, neurobiology, and neuroimaging studies furthermore.

Data Availability

The data utilized to support the findings are available from the corresponding authors upon request.

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' Contributions

Chen PA and Wang HY contributed equally to this work.

Acknowledgments

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Supplementary Materials

The study procedure was shown in Supplement Figure 1, and the mouse movement tracking was shown in Supplement Figure 2. The parameters of the study measurement including central frequencies, central duration percentages, central travelled speed, and overall travelled speed were shown in Supplement Figures 3–6. Supplements Figures 7 and8 demonstrated the behavioral gaiting following risperidone and valproic acid treatment, respectively. *(Supplementary Materials)*

References

[1] M. Garriga, I. Pacchiarotti, S. Kasper et al., "Assessment and management of agitation in psychiatry: expert consensus," *The World Journal of Biological Psychiatry*, vol. 17, no. 2, pp. 86–128, 2016.

[2] C. M. Cornaggia, M. Beghi, F. Pavone, and F. Barale, "Aggression in psychiatry wards: a systematic review," *Psychiatry Research*, vol. 189, no. 1, pp. 10–20, 2011.

[3] J. W. Newcomer, N. B. Farber, and J. W. Olney, "NMDA receptor function, memory, and brain aging," *Dialogues in Clinical Neuroscience*, vol. 2, no. 3, pp. 219–232, 2000.

[4] D. Cadinu, B. Grayson, G. Podda, M. K. Harte, N. Doostdar, and J. C. Neill, "NMDA receptor antagonist rodent models for cognition in schizophrenia and identification of novel drug treatments, an update," *Neuropharmacology*, vol. 142, pp. 41–62, 2018.

[5] S. Ham, T. K. Kim, S. Chung, and H. I. Im, "Drug abuse and psychosis: new insights into drug-induced psychosis," *Experimental Neurobiology*, vol. 26, no. 1, pp. 11–24, 2017.

[6] M. L. Carlsson, A. Carlsson, and M. Nilsson, "Schizophrenia: from dopamine to glutamate and back," *Current Medicinal Chemistry*, vol. 11, no. 3, pp. 267–277, 2004.

[7] H. Grunze, E. Vieta, G. M. Goodwin et al., "The World Federation of Societies of Biological Psychiatry (WFSBP) guidelines for the biological treatment of bipolar disorders: acute and long-term treatment of mixed states in bipolar disorder," *The World Journal of Biological Psychiatry*, vol. 19, no. 1, pp. 2–58, 2018.

[8] H. Grunze, E. Vieta, G. M. Goodwin et al., "The World Federation of Societies of Biological Psychiatry (WFSBP) guidelines for the biological treatment of bipolar disorders: update 2009 on the treatment of acute mania," *The World Journal of Biological Psychiatry*, vol. 10, no. 2, pp. 85–116, 2009.

[9] G. M. Goodwin, P. M. Haddad, I. N. Ferrier et al., "Evidence-based guidelines for treating bipolar disorder: revised third edition recommendations from the British Association for Psychopharmacology," *Journal of Psychopharmacology*, vol. 30, no. 6, pp. 495–553, 2016.

[10] L. N. Yatham, S. H. Kennedy, S. V. Parikh et al., "Canadian Network for Mood and Anxiety Treatments (CANMAT) and International Society for Bipolar Disorders (ISBD) 2018 guidelines for the management of patients with bipolar disorder," *Bipolar Disorders*, vol. 20, no. 2, pp. 97–170, 2018.

[11] M. Kokkinou, A. H. Ashok, and O. D. Howes, "The effects of ketamine on dopaminergic function: meta-analysis and review of the implications for neuropsychiatric disorders," *Molecular Psychiatry*, vol. 23, no. 1, pp. 59–69, 2018.

[12] J. Eldufani, A. Nekoui, and G. Blaise, "Nonanesthetic effects of ketamine: a review article," *The American Journal of Medicine*, vol. 131, no. 12, pp. 1418–1424, 2018.

[13] M. Haaf, G. Leicht, S. Curic, and C. Mulert, "Glutamatergic deficits in schizophrenia-biomarkers and pharmacological interventions within the ketamine model," *Current Pharmaceutical Biotechnology*, vol. 19, no. 4, pp. 293–307, 2018.

[14] Y. Liu, D. Lin, B. Wu, and W. Zhou, "Ketamine abuse potential and use disorder," *Brain Research Bulletin*, vol. 126, pp. 68–73, 2016.

[15] G. Lee and Y. Zhou, "NMDAR hypofunction animal models of schizophrenia," *Frontiers in Molecular Neuroscience*, vol. 12, p. 185, 2019.

[16] L. Prut and C. Belzung, "The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review," *European Journal of Pharmacology*, vol. 463, no. 1-3, pp. 3–33, 2003.

[17] Y. C. Chen, F. M. Tsai, M. L. Chen, and M. Carotenuto, "Antipsychotic drugs reverse MK801-inhibited cell migration and F-actin condensation by modulating the rho signaling pathway in B35 cells," *Behavioural Neurology*, vol. 2020, 9 pages, 2020.

[18] E. L. Hargreaves and D. P. Cain, "MK801-induced hyper activity: duration of effects in rats," *Pharmacology, Biochemistry, and Behavior*, vol. 51, no. 1, pp. 13–19, 1995.

[19] Y. A. Su, T. M. Si, D. F. Zhou et al., "Risperidone attenuates MK-801-induced hyperlocomotion in mice via the blockade of serotonin 5-HT 2A/2C receptors," *European Journal of Pharmacology*, vol. 564, no. 1-3, pp. 123–130, 2007.

[20] K. Ito, T. Abekawa, and T. Koyama, "Valproate blocks high-dose methamphetamine-induced behavioral cross-sensitization to locomotion-inducing effect of dizocilpine (MK-801), but not methamphetamine," *Psychopharmacology*, vol. 186, no. 4, pp. 525–533, 2006.

[21] D. E. Casey, D. G. Daniel, A. A. Wassef, K. A. Tracy, P. Wozniak, and K. W. Sommerville, "Effect of divalproex combined with olanzapine or risperidone in patients with an acute exacerbation of schizophrenia," *Neuropsychopharmacology*, vol. 28, no. 1, pp. 182–192, 2003.

[22] D. L. Kelly, R. R. Conley, S. Feldman, Y. Yu, R. P. McMahon, and C. M. Richardson, "Adjunct divalproex or lithium to clozapine in treatment-resistant schizophrenia," *The Psychiatric Quarterly*, vol. 77, no. 1, pp. 81–95, 2006.

[23] T. Suzuki, H. Uchida, H. Takeuchi et al., "Augmentation of atypical antipsychotics with valproic acid. An open-label study for most difficult patients with schizophrenia," *Human Psychopharmacology*, vol. 24, no. 8, pp. 628–638, 2009.

[24] P. T. Tseng, Y. W. Chen, W. Chung et al., "Significant effect of valproate augmentation therapy in patients with schizophrenia: a meta-analysis study," *Medicine*, vol. 95, no. 4, article e2475, 2016.

[25] Y. Wang, J. Xia, B. Helfer, C. Li, and S. Leucht, "Valproate for schizophrenia," *Cochrane Database of Systematic Reviews*, vol. 2016, no. 11, article CD004028, 2016.

[26] A. Hasan, P. Falkai, T. Wobrock et al., "World Federation of Societies of Biological Psychiatry (WFSBP) guidelines for biological treatment of schizophrenia, part 1: update 2012 on the acute treatment of schizophrenia and the management of treatment resistance," *The World Journal of Biological Psychiatry*, vol. 13, no. 5, pp. 318–378, 2012.

[27] E. Horowitz, L. C. Bergman, C. Ashkenazy, I. Moscona-Hurvitz, H. Grinvald-Fogel, and R. Magnezi, "Off-label use of sodium valproate for schizophrenia," *PLoS One*, vol. 9, no. 3, article e92573, 2014.

[28] A. D. Forrest, C. A. Coto, and S. J. Siegel, "Animal models of psychosis: current state and future directions," *Current Behavioral Neuroscience Reports*, vol. 1, no. 2, pp. 100–116, 2014.

[29] D. K. E. Beyer and N. Freund, "Animal models for bipolar disorder: from bedside to the cage," *International Journal of Bipolar Disorders*, vol. 5, no. 1, p. 35, 2017.

[30] M. Irifune, T. Shimizu, and M. Nomoto, "Ketamine-induced hyperlocomotion associated with alteration of presynaptic components of dopamine neurons in the nucleus accumbens of mice," *Pharmacology, Biochemistry, and Behavior*, vol. 40, no. 2, pp. 399–407, 1991.

[31] K. A. Clarke and J. Still, "Gait analysis in the mouse," *Physiology & Behavior*, vol. 66, no. 5, pp. 723–729, 1999.

[32] J. Svoboda, A. Stankova, M. Entlerova, and A. Stuchlik, "Acute administration of MK-801 in an animal model of psychosis in rats interferes with cognitively demanding forms of behavioral flexibility on a rotating arena," *Frontiers in Behavioral Neuroscience*, vol. 9, p. 75, 2015.

[33] F. J. van der Staay, K. Rutten, C. Erb, and A. Blokland, "Effects of the cognition impairer MK-801 on learning and memory in mice and rats," *Behavioural Brain Research*, vol. 220, no. 1, pp. 215–229, 2011.

[34] F. J. Barbosa, B. Hesse, R. B. de Almeida, I. P. Baretta, R. Boerngen-Lacerda, and R. Andreatini, "Magnesium sulfate and sodium valproate block methylphenidate-induced hyperlocomotion, an animal model of mania," *Pharmacological Reports*, vol. 63, no. 1, pp. 64–70, 2011.

[35] T. Abekawa, K. Ito, S. Nakagawa, Y. Nakato, and T. Koyama, "Olanzapine and risperidone block a high dose of methamphetamine-induced schizophrenia-like behavioral abnormalities and accompanied apoptosis in the medial prefrontal cortex," *Schizophrenia Research*, vol. 101, no. 1-3, pp. 84–94, 2008.

[36] Z. Rogoz and K. Kaminska, "The effect of combined treatment with escitalopram and risperidone on the MK-801-induced changes in the object recognition test in mice," *Pharmacological Reports*, vol. 68, no. 1, pp. 116–120, 2016.

[37] V. Bubenikova, M. Votava, J. Horacek, T. Palenicek, and C. Dockery, "The effect of zotepine, risperidone, clozapine and olanzapine on MK-801-disrupted sensorimotor gating," *Pharmacology, Biochemistry, and Behavior*, vol. 80, no. 4, pp. 591–596, 2005.

[38] T. N. Douma, M. J. Millan, P. M. Verdouw, R. S. Oosting, B. Olivier, and L. Groenink, "Valproate improves prepulse inhibition deficits induced by corticotropin-releasing factor independent of GABA (A) and GABA (B) receptor activation," *Neuropharmacology*, vol. 79, pp. 66–74, 2014.

[39] K. Merritt, A. Egerton, M. J. Kempton, M. J. Taylor, and P. K. McGuire, "Nature of glutamate alterations in schizophrenia: a meta-analysis of proton magnetic resonance spectroscopy studies," *JAMA Psychiatry*, vol. 73, no. 7, pp. 665–674, 2016.

[40] A. M. Bradford, K. M. Savage, D. N. Jones, and M. Kalinichev, "Validation and pharmacological characterisation of MK-801-induced locomotor hyperactivity in BALB/C mice as an assay for detection of novel antipsychotics," *Psychopharmacology*, vol. 212, no. 2, pp. 155–170, 2010.

[41] M. Chatterjee, R. Verma, S. Ganguly, and G. Palit, "Neurochemical and molecular characterization of ketamine-

induced experimental psychosis model in mice," *Neuropharmacology*, vol. 63, no. 6, pp. 1161–1171, 2012.

[42] I. Braun, J. Genius, H. Grunze, A. Bender, H. J. Moller, and D. Rujescu, "Alterations of hippocampal and prefrontal GABAergic interneurons in an animal model of psychosis induced by NMDA receptor antagonism," *Schizophrenia Research*, vol. 97, no. 1-3, pp. 254–263, 2007.

[43] M. W. Tyler, H. B. Yourish, D. F. Ionescu, and S. J. Haggarty, "Classics in chemical neuroscience: ketamine," *ACS Chemical Neuroscience*, vol. 8, no. 6, pp. 1122–1134, 2017.

[44] P. Kovacic and R. Somanathan, "Clinical physiology and mechanism of dizocilpine (MK-801): electron transfer, radicals, redox metabolites and bioactivity," *Oxidative Medicine and Cellular Longevity*, vol. 3, no. 1, pp. 13–22, 2010.

[45] M. Carlsson and A. Carlsson, "Interactions between glutamatergic and monoaminergic systems within the basal ganglia–implications for schizophrenia and Parkinson's disease," *Trends in Neurosciences*, vol. 13, no. 7, pp. 272–276, 1990.

[46] A. A. Wassef, S. G. Dott, A. Harris et al., "Randomized, placebo-controlled pilot study of divalproex sodium in the treatment of acute exacerbations of chronic schizophrenia," *Journal of Clinical Psychopharmacology*, vol. 20, no. 3, pp. 357–361, 2000.

[47] D. E. Casey, D. G. Daniel, C. Tamminga et al., "Divalproex ER combined with olanzapine or risperidone for treatment of acute exacerbations of schizophrenia," *Neuropsychopharmacology*, vol. 34, no. 5, pp. 1330–1338, 2009.

[48] W. Löscher, "Valproate: a reappraisal of its pharmacodynamic properties and mechanisms of action," *Progress in Neurobiology*, vol. 58, no. 1, pp. 31–59, 1999.

[49] W. Löscher, "Basic pharmacology of Valproate," *CNS Drugs*, vol. 16, no. 10, pp. 669–694, 2002.

[50] L. C. Johannessen, "Antiepileptic drugs in non-epilepsy disorders: relations between mechanisms of action and clinical efficacy," *CNS Drugs*, vol. 22, no. 1, pp. 27–47, 2008.

[51] A. Rocha, N. Hart, and K. A. Trujillo, "Differences between adolescents and adults in the acute effects of PCP and ketamine and in sensitization following intermittent administration," *Pharmacology, Biochemistry, and Behavior*, vol. 157, pp. 24–34, 2017.

[52] M. L. S. Bates and K. A. Trujillo, "Long-lasting effects of repeated ketamine administration in adult and adolescent rats," *Behavioural Brain Research*, vol. 369, article 111928, 2019.

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Research Article

The Medial Prefrontal Cortex, Nucleus Accumbens, Basolateral Amygdala, and Hippocampus Regulate the Amelioration of Environmental Enrichment and Cue in Fear Behavior in the Animal Model of PTSD

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A growing body of evidence showed that environmental enrichment (EE) ameliorated footshock-induced fear behavior of posttraumatic stress disorder (PTSD). However, no research comprehensively tested the effect of EE, cue, and the combination of EE and cue in footshock-induced fear behavior of PTSD symptoms. The present study addressed this issue and examined whether the medial prefrontal cortex (mPFC, including the cingulate cortex 1 (Cg1), prelimbic cortex (PrL), and infralimbic cortex (IL)), the nucleus accumbens (NAc), the basolateral amygdala (BLA), and the hippocampus (e.g., CA1, CA3, and dentate gyrus (DG)) regulated the amelioration of the EE, cue, or the combination of EE and cue. The results showed that EE or cue could reduce fear behavior. The combination of EE and cue revealed a stronger decrease in fear behavior. The cue stimulus may play an occasion setting or a conditioned stimulus to modulate the reduction in fear behavior induced by footshock. Regarding the reduction of the EE in fear behavior, the Cg1 and IL of the mPFC and the NAc upregulated the c-Fos expression; however, the BLA downregulated the c-Fos expression. The mPFC (i.e., the Cg1, PrL, and IL) and the hippocampus (i.e., the CA1, CA3, and DG) downregulated the c-Fos expression in the suppression of the cue in fear behavior. The interaction of EE and cue in reduction of fear behavior occurred in the Cg1 and NAc for the c-Fos expression. The data of c-Fos mRNA were similar to the findings of the c-Fos protein expression. These findings related to the EE and cue modulations in fear behavior may develop a novel nonpharmacological treatment in PTSD.

1. Introduction

Posttraumatic stress disorder (PTSD) is a complicated mental illness [1]. PTSD individuals often encounter a variety of psychiatric symptoms that include feeling fear, helplessness, horror, increased arousal, autonomic hyperarousal activities, fear sensitization, serious startle responses, hypervigilance, insomnia, irritability, and impaired concentration [2–4]. Concerning the numerous symptoms of PTSD, the fear symptom through classical conditioning to form fear memory in the animal model of PTSD effectively simulated the PTSD's human symptoms [5]. Moreover, PTSD's fear symptom is crucial to the etiology of PTSD, and it is highly preserved in the process of evolution cross-species [5].

In light of the previous studies, environmental enrichment (EE) exposures can effectively reduce the PTSD symptoms in the animal model [6–11]. For example, EE reduced anxiety due to differentially activating the serotonin system and neuropeptide system in the PTSD animal model [11]. Chronic EE exposures reduced neonatal isolation-induced contextual freezing; however, chronic EE exposures did not affect single prolonged stress-related to anxiety behaviors or analgesia

[9]. Acute EE exposures facilitated fear extinction through neuropeptide Y-Y1 receptor modulation in the hippocampus [10]. EE enhanced anxiolytic effect induced by deep-brain stimulation in the medial prefrontal cortex (mPFC) in the PTSD animal model [6]. Traumatic stress caused the reduction in the volume of the hippocampus and central amygdala, and EE was able to ameliorate trauma-induced volume decreases of the hippocampus resulted in behavioral, morphological, and molecular changes [8]. EE facilitated the negative feedback of the hypothalamus-pituitary-adrenal gland (HPA) axis-related stress system and then improved abnormal behaviors through decreasing the glucocorticoid receptors in the hypothalamus and hippocampus [7]. Therefore, EE is a nonpharmacological treatment in reducing PTSD symptoms at behavioral, neuronal, and molecular levels.

The EE manipulation combined exposure of sensory, physical, cognitive, and social stimuli in the contextual surroundings for individuals, and the EE simulated a contextual environment. Animals exposed the entire components of sensory, physical, social, and cognitive stimuli [12–14]. In contrast to the EE stimuli, the cue is a single component or stimulus of the contextual stimuli [15]. Based on the previous evidence, the different category of fear conditioning is controlled by the different neural substrates: the hippocampus is contributable to the context-induced fear conditioning [15, 16], whereas the amygdala modulates the cue-induced fear conditioning [17, 18]. The hippocampus plays a function in configural memory and the encoding process of contextual stimulus [19]. The amygdala is essential to regulate the negative valence of emotion and negative emotional processes [20]. Recently, accumulated studies have clarified the roles of the mPFC, amygdala, and hippocampus in the fear conditioning of the PTSD symptoms after encountering a traumatic stress event [21–24]. The individual who experienced traumatic events was revealed to decrease the volumes of the hippocampus and anterior cingulate cortex; however, it increased the hyperactivity of the amygdala; moreover, the mPFC showed dysfunction that cannot inhibit the activity of the amygdala [21]. The hippocampus contributed to spatial learning and declarative memory; moreover, the hippocampus was projected from the amygdala, and thereby, the hippocampus-related functions, including spatial learning and memory, were regulated by the amygdala's activity [25]. Alternatively, little research has demonstrated that the nucleus accumbens (NAc) might be involved in the PTSD symptoms associated with stress behaviors [26, 27]. Therefore, the study examined whether the mPFC, amygdala, hippocampus, and NAc mediated fear behavior of PTSD symptoms.

Altogether, some critical issues should be concerned: (a) Whether EE decreased fear symptoms of PTSD. (b) Whether a stimulus cue can reduce PTSD fear symptoms. (c) Using the c-Fos labeling and the assessment of c-Fos mRNA expression with the qRT-PCR methods, whether the subareas of mPFC (e.g., the Cg1, PrL, and IL), the subregions of the hippocampus (e.g., the CA1, CA3, and DG), the NAc, and BLA were involved in the amelioration of EE, cue, or the EE and cue combination in footshock-induced fear behavior of the PTSD symptom.

2. Methods and Materials

2.1. Animals. Fifty-six male wild-type C57BL/6 mice (approximate 25~35 g at the beginning of the experiments) were bought from National Laboratory for Animal Breeding and Research Center, Taipei, Taiwan. All mice were randomly assigned into no environmental enrichment (no EE; i.e., standard housing) and EE cages. The mice of no EE and EE groups were raised with another two mice in a colony room with constant temperature (approximately 23 ± 2°C) and light-dark cycle (light on 6 : 00-18 : 00). The EE cage is a plastic surrounding shell box, which is 33.5 cm long × 25.0 cm wide × 28.8 cm high. The standard cage is a plastic box, and its surrounding size is 30.0 cm long × 18.8 cm wide × 13.5 cm high. Food and water were provided ad libitum. The experiments were performed in compliance with the American Psychological Association ethical standards for the treatment of animals. A description of the treatment details was submitted and received approval from the Institutional Animal Care and Use Committee (IACUC) of Fo Guang University. Every effort was made to minimize the animals' suffering and the number of animals used.

2.2. Apparatus

2.2.1. Inescapable Footshock. The inescapable footshock apparatus is a box composed of a plastic surrounding shell measuring 60 cm × 60 cm × 72 cm high. The apparatus floor is composed of metal grids (0.3 cm diameter at 0.7 cm grid intervals). On the footshock procedure, mice were placed in the footshock apparatus for 2 min. Then, mice were given a 2 mA footshock (duration, 10 seconds), and the other mice received no footshock at their home cage [28].

2.3. Behavioral Procedure. All mice received an adaptation regimen for ad libitum food and water for seven days in the adaptation phase. After that, all mice were dependent on their assigned groups, and they were, respectively, raised in a mixed environment, including any combinations of no EE, EE, no cue, and cue until the end of the experiment (day 0-day 20). On day 17, all mice were given a single footshock (2 mA, 10 seconds) in the footshock apparatus. On days 18-20, all mice received the situational reminder procedure. Note, the current rate of footshock (2 mA, 10 seconds) and three freezing-times in situational reminders were determined by our previous studies [22, 29]. The mice were placed in the footshock apparatus for 2 min without footshock. One hundred twenty minutes after completing the last behavioral test, the immunohistochemical staining and the qRT-PCR method were, respectively, performed to measure c-Fos protein and c-Fos mRNA expression in the selected brain areas (see Figure 1).

All of the mice were assigned into EE or cue environments into four groups: no EE/no Cue, no EE/cue, EE/no cue, and EE/cue groups ($n = 6$, per group). Regarding the housing condition, the mice in the no EE/No cue group were given no EE and no cue in the standard cage. The no EE/cue group mice were given no EE with a cue stimulus in the standard cage. The mice in the EE/no cue group were given an EE procedure without a cue stimulus. The mice in the EE/

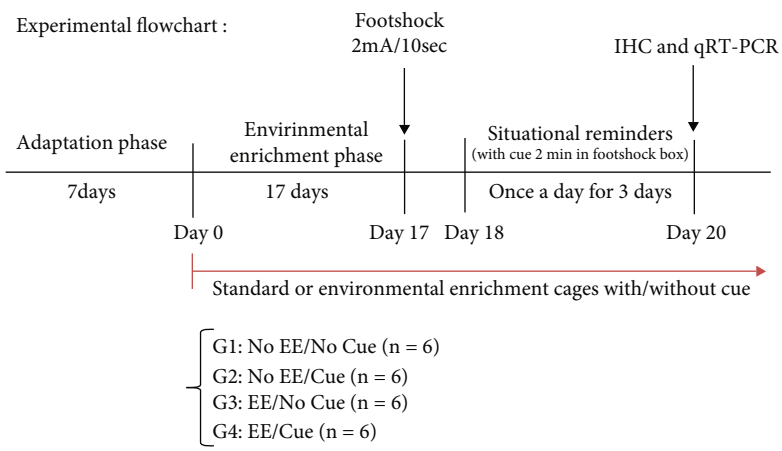

Figure 1: Overview of the experimental procedures. The behavioral processes were shown in the adaptation, EE phase, footshock treatment, and situational reminder phases. On days 0-17, the mice in the EE groups were subjected to the EE procedure. These mice received the EE procedure at the end of the experiment. However, the mice in the no EE groups were housed in the standard cage. On day 17, all mice were placed in the footshock box for 2 min, and then the mice were subjected to the footshock treatment (2 mA, 10 seconds). On days 18-20, all mice have given a situational reminder procedure that the mice were placed in the footshock box for 2 min with the cue ball but not any footshock. One hundred twenty minutes after the last behavioral test, the immunohistochemical staining with the c-Fos protein expression on day 20. All mice were assigned to the no EE/no cue, no EE/cue, EE/no cue, and EE/cue groups ($n = 6$, per group). EE: environmental enrichment.

cue group were given an EE procedure with a cue stimulus. The EE cage included two large wooden blocks, a shelter, a retreat, a tunnel, crow ball, motor running wheel, and bone toys, and the EE procedure was followed the previous study [30]. The cue stimulus was designed for the mice exposed to a plastic round ball (diameter is 3 cm) in the EE or standard cages for 24 hours. This cue stimulus, a white round ball, is different in size and texture from the crow ball of the EE cage.

2.4. Immunohistochemical Staining. Mice were sacrificed by sodium pentobarbital overdose after completing behavioral tests for 120 min (i.e., labeling IHC c-Fos for the best expression). When completely unresponsive, mice were perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M sodium phosphate-buffered saline (PBS). The brain tissues were dissected, blocked, postfixed for 3 days, and transferred to 30% sucrose for cryoprotection for 2 days until the specimens sink to the bottom of the solution. Forty-micron coronal sections were cut on freezing using a sliding microtome. All brain slices were processed by c-Fos immunoreactivity staining. Free-floating brain slices were washed once for 10 mins in 0.1 M PBS, permeabilized in 3% H_2O_2 for 1 hour, washed three times in PBS for 10mins, and then soaked in normal goat serum for 1 hour. After washing with PBS once for 10 mins, the slices were incubated overnight with the specific first antibody (i.e., sheep anti-c-Fos primary antibody at a dilution of 1 : 500). This antibody was raised against a peptide sequence to enable crossreaction with other proteins for detecting c-Fos immunoreactivity. The slices were then washed once with PBS for 10 mins, and then, these slices were incubated in a second antibody (i.e., biotinylated anti-sheep secondary antibody at a dilution of 1 : 500) for 2 hours. Ten minutes with PBS washing later, the bound secondary antibody was amplified using the Vector Elite ABC kit.

The positive expression nucleus of neurons was quantified for the selected brain areas. Counting was performed visually at ×20 magnification by a researcher blinded to the condition of each mouse. Every third slice of the brain tissue was selected into an available counted section. The software ImageJ was used to count the amounts of c-Fos-positive neurons [31].

2.5. Real-Time Quantitative PCR of c-Fos. Based on the manufacturer's instructions, total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). For cDNA synthesis, total RNA was applied with random hexamers. Later, the reverse-transcription PCR was the amplification of *c-fos*. For the amplification, it was initiated with a pair of *c-fos* primers (forward: 5′-TCCACTGCCTGGGACA GAA-3′; reverse: 5′-CGCAGCGATCTTCATCAAAC-3′) with a volume of 20 μl. For undergoing the process, it was a denaturation stage at 95°C for 10 minutes. After that, 28 cycles of denaturation were conducted at 95°C for one minute. The primer was annealing at 55°C for 30 seconds and extension at 72°C for 45 seconds. The cycling steps were completed, and the final extension was at 72°C for five minutes. The reactions were repeated three times and were performed in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Thermo Fisher Scientific, USA). The mean expression levels of the housekeeping gene were with a pair of beta-actin primers (forward: 5′CAACTT GATGTATGAAGGCTTTGGT-3′; reverse: 5′-ACTTTT ATTGGTCTCAAGTCAGTGTACAG-3′). It was used as the internal control to normalize the variability of *c-fos* expression levels. Relative changes in the gene expression were measured using the $2^{-\Delta\Delta CT}$ method [32].

Table 1: Mean (± SEM) freezing time (sec.) was analyzed by a 2 × 2 × 3 three-way mixed analysis of variance (ANOVA) (environmental enrichment vs. cue vs. session).

2 × 3 × 2 three-way mixed ANOVA	
Environmental enrichment	$F_{1,20} = 70.73$, $p < 0.05^*$
Cue	$F_{1,20} = 14.14$, $p < 0.05^*$
Session	$F_{2,40} = 27.95$, $p < 0.05^*$
Environmental enrichment × cue	$F_{1,20} = 3.25$, $p > 0.05$
Environmental enrichment × session	$F_{2,40} = 2.11$, $p > 0.05$
Cue × session	$F_{2,40} = 0.28$, $p > 0.05$
Environmental enrichment × cue × session	$F_{2,40} = 0.89$, $p > 0.05$

$^*p < 0.05$, significant difference.

2.6. Statistical Analysis. A three-way mixed (environmental enrichment vs. cue vs. session) analysis of variance (ANOVA) was performed for the freezing time. One-way ANOVA was conducted for the total freezing time in the main effect of environmental enrichment, cue, and situational reminders. Furthermore, two-way mixed (group vs. session) ANOVA was performed for the freezing time over sessions 1-3 in situational reminders and total freezing time among the no EE/no cue, no EE/cue, EE/no cue, and EE/Cue groups. When appropriate, the post hoc with Tukey's Honest Significant Difference (HSD) test was performed.

Regarding the examination of the c-Fos protein expression and c-Fos mRNA expression, two-way ANOVA analysis was conducted, and one-way ANOVA analysis was performed. For c-Fos protein expression analysis, the selected brain areas were determined, including Cg1, PrL, IL, NAc, BLA, CA1, CA2, and DG. For c-Fos mRNA expression analysis, the mPFC, NAc, amygdala, and hippocampus were determined. When appropriate, the post hoc with Tukey's HSD test was performed. $^*p < 0.05$ was considered statistically significant compared to the no EE/no cue group. $\#p < 0.05$ was considered statistically significant compared to the no EE/cue group. $\$p < 0.05$ was considered statistically significant compared to the EE/no cue group.

3. Results

3.1. PTSD Behavioral Tests. To examine the effect of environmental enrichment and cue in freezing behavior of the PTSD animal model, a 2 × 2 × 3 three-way mixed ANOVA analysis indicated that significant differences occurred in the factor of EE ($F(1, 20) = 70.73$, $p < 0.05$), cue ($F(1, 20) = 14.14$, $p < 0.05$), and session ($F(2, 40) = 27.95$, $p < 0.05$). Nonsignificant differences occurred in the interaction of EE and cue ($F(1, 20) = 3.24$, $p > 0.05$), the interaction of EE and session ($F(2, 40) = 2.11$, $p > 0.05$), the interaction of cue and session ($F(2, 40) = 0.28$, $p > 0.05$), and the interaction of EE, cue, and session ($F(2, 40) = 0.89$, $p > 0.05$). The results indicated that EE, cue, and session had significant differences (Table 1). Furthermore, one-way ANOVA analysis was performed. The results showed that the main effect of EE was significantly decreased in total freezing time ($F(1, 22) = 41.62$, $p < 0.05$; Figure 2). The main effect of cue was seemingly decreased in total freezing time ($F(1, 22) = 3.21$, $p = 0.08$; Figure 3). The main effect of session was significantly decreased from session 1 to session 3 ($F(2, 46) = 27.61$, $p < 0.05$; Figure 4). The post hoc Tukey's HSD appeared that total freezing time of session 2 and 3 was significantly decreased when compared to session 1 ($p < 0.05$). The total freezing time of session 3 was significantly decreased than that of session 2 ($p < 0.05$). In summary, total freezing time was significantly decreased as manipulations of EE, cue, and sessions.

Alternatively, a 4 × 3 mixed two-way ANOVA analysis was conducted for freezing behavior. The results showed that significant differences occurred in the factor of group ($F(3, 20) = 29.37$, $p < 0.05$) and session ($F(2, 40) = 27.95$, $p < 0.05$). However, there was a nonsignificant difference in the interaction of group and session ($F(6, 40) = 1.09$, $p > 0.05$). Post hoc with Tukey's HSD tests showed that the freezing time in the no EE/cue, EE/no cue, and EE/cue groups was seemingly decreased compared to the no EE/no cue group in sessions 1 and 2 ($p < 0.05$). In session 3, the freezing time in the no EE/cue, EE/no cue, and EE/cue groups was significantly decreased than that of the no EE/no cue group ($p < 0.05$); moreover, the EE/no cue and EE/cue groups were significantly decreased compared to the no EE/cue group ($p < 0.05$). Therefore, the combination of EE and cue exhibited the lowest freezing time. EE or cue manipulations could suppress freezing behavior. The EE manipulation likely reduced much more freezing time when compared to the cue manipulation (Figure 5).

To analyze mean (± SEM) total freezing time that was merged with the behavioral data overall sessions 1-3, two-way ANOVA was conducted to show that significant differences occurred in the factor of EE ($F(1, 20) = 70.73$, $p < 0.05$), cue ($F(1, 20) = 14.14$, $p < 0.05$), and the interaction of EE and cue ($F(1, 20) = 3.25$, $p < 0.05$). Furthermore, one-way ANOVA indicated that the factor of group was significant differences ($F(3, 20) = 29.37$, $p < 0.05$). Post hoc Tukey's HSD tests showed that the no EE/cue, EE/no cue, and EE/cue were significantly decreased in total freezing time compared to the no EE/no cue group ($p < 0.05$). The total freezing time of the EE/no cue and EE/cue group was significantly decreased than that of the no EE/cue group ($p < 0.05$). However, the freezing time of the EE/no cue group was not significantly different from that of the EE/cue group ($p > 0.05$; Figure 6). The similar evidence as

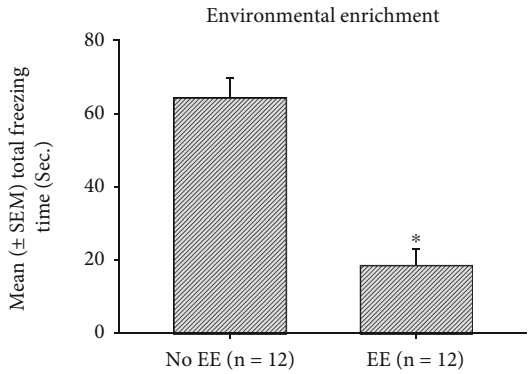

Figure 2: Assessments of the mean (± SEM) total freezing time (Sec.) for the min effect of EE in the no EE ($n = 12$) and EE ($n = 12$) conditions. EE: environmental enrichment. $^{*}p < 0.05$ indicates significant differences compared to the no EE condition.

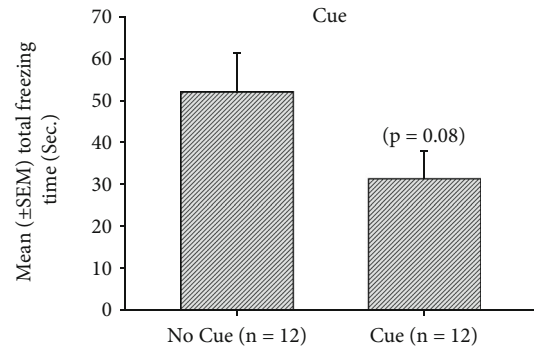

Figure 3: Assessments of mean (± SEM) total freezing time (Sec.) for the main effect of the cue in the no cue ($n = 12$) and cue ($n = 12$) conditions. $^{*}p < 0.05$ indicates significant differences compared to the no cue condition.

mentioned above that the EE manipulation might be effective to reduce total freezing time. The combination of EE and cue treatments was likely the greatest reduction in total freezing behavior.

3.2. Immunohistochemical Staining with c-Fos Protein Expression. To examine the involvements of selected brain areas in the EE, cue, or the interaction of EE and cue, a 2 × 2 two-way ANOVA was conducted for the c-Fos expression after testing freezing behavior. The results of the EE manipulation showed that the c-Fos expression was significant increases in Cg1, IL, and NAc but decreases in BLA compared to the no EE manipulation ($p < 0.05$); the nonsignificant c-Fos expression occurred in the PrL, CA1, CA3, and DG ($p > 0.05$). The cue manipulations showed that the c-Fos expression was significant decreases in the Cg1, PrL, IL, CA1, CA3, and DG compared to the no cue manipulation ($p < 0.05$); nonsignificant differences occurred in the NAc and BLA ($p > 0.05$). The EE and cue interactions were significant differences in the C1g and NAc ($p < 0.05$); however, the other brain areas did not show significant differences ($p > 0.05$; Table 2).

On the other hand, one-way ANOVA was conducted to analyze the c-Fos expression in the selected brain areas. The results showed that a significant difference occurred in the factor of the group ($F(3, 12) = 29.35$, $p < 0.05$). In post hoc Tukey's HSD tests, the Cg1 showed that the EE/no cue was significantly increased in the c-Fos expression than the no EE/no cue and no EE/cue groups ($p < 0.05$); the no EE/cue and EE/cue were significantly decreased in the c-Fos expression compared to the no EE/no cue group ($p < 0.05$); the EE/cue was significantly decreased in the c-Fos expression than the EE/cue group ($p < 0.05$; Figure 7(a)).

In the PrL c-Fos expression measurements, a significant difference occurred in the factor of group ($F(3, 12) = 5.21$, $p < 0.05$). In post hoc Tukey's HSD tests, the results showed that the EE/no cue was significantly increased in the c-Fos expression than the no EE/cue group ($p < 0.05$; Figure 7(b)).

In the IL c-Fos expression measurements, a significant difference occurred in the factor of group ($F(3, 12) = 4.44$, $p < 0.05$). In post hoc Tukey's HSD tests, the results showed that the EE/no cue was significantly increased in the c-Fos expression than the no EE/cue group ($p < 0.05$; Figures 7(c) and 8).

In the NAc c-Fos expression measurements, a significant difference occurred in the factor of group ($F(3, 12) = 4.18$, $p < 0.05$). In post hoc Tukey's HSD tests, the results showed that the EE/no cue was significantly increased in the c-Fos expression than the no EE/no cue group ($p < 0.05$; Figure 9(a)).

In the BLA c-Fos expression measurements, a significant difference occurred in the factor of the group ($F(3, 12) = 4.32$, $p < 0.05$). In post hoc Tukey's HSD tests, the results showed that the EE/cue was significantly decreased in the c-Fos expression than the No EE/no cue group ($p < 0.05$; Figures 9(b) and 10).

In the CA1 c-Fos expression assessments, a significant difference occurred in the factor of group ($F(3, 12) = 11.40$, $p < 0.05$). In post hoc Tukey's HSD tests, the results showed that the no EE/cue and EE/cue groups were significantly decreased in the c-Fos expression than the no EE/no cue group ($p < 0.05$); the EE/no cue and EE/cue were significantly increased and decreased in the c-Fos expression, respectively, compared to the no EE/cue group ($p < 0.05$; Figure 11(a)).

In the CA3 c-Fos expression assessments, a significant difference occurred in the factor of group ($F(3, 12) = 5.81$, $p < 0.05$). In post hoc Tukey's HSD tests, the results showed that the no EE/cue group was significantly decreased in the c-Fos expression than the no EE/no cue group ($p < 0.05$); the EE/no cue was significantly increased in the c-Fos expression compared to the no EE/cue group ($p < 0.05$; Figure 11(b)).

In the DG c-Fos expression assessments, a significant difference occurred in the factor of group ($F(3, 12) = 6.89$, $p < 0.05$]. In post hoc Tukey's HSD tests, the results showed that the EE/no cue group was significantly increased in the c-Fos expression than the no EE/cue group ($p < 0.05$); the EE/cue was significantly decreased in the c-Fos expression compared to the EE/no cue group ($p < 0.05$; Figures 11(c) and 12).

Therefore, the Cg1, IL, and NAc were upregulated in the c-Fos expression, but the BLA was a downregulation in the

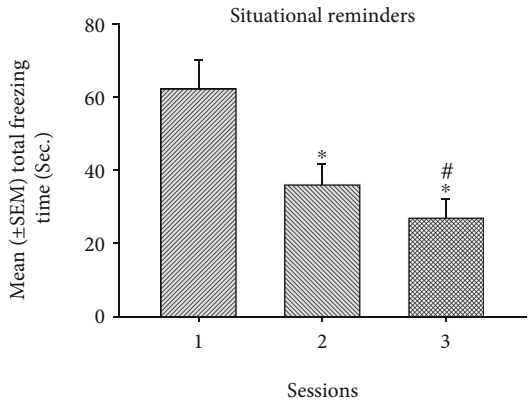

Figure 4: The mean (± SEM) total freezing time (Sec.) in the situational reminders for sessions 1-3. $^{*}p < 0.05$ indicates significant differences compared to session 1. $\#p < 0.05$ indicates significant differences compared to session 2.

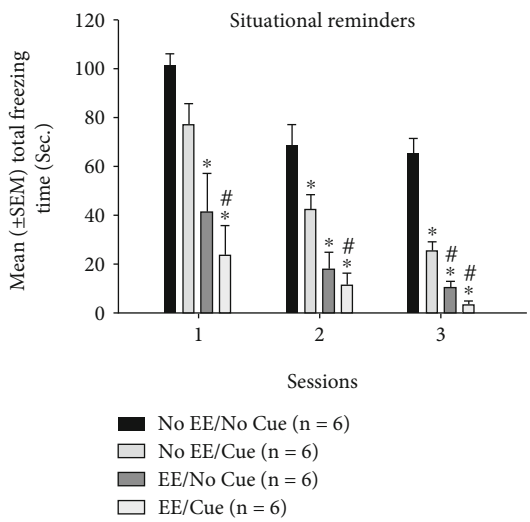

Figure 5: Mean (± SEM) freezing time for the situational reminder in sessions 1-3 for the no EE/no cue, no EE/cue, EE/no cue, and EE/cue groups ($n = 6$, per group). EE: environmental enrichment. $^{*}p < 0.05$ indicates significant differences compared to the no EE/no cue group; $\#p < 0.05$ indicates significant differences compared to the no EE/cue group.

c-Fos expression through the EE manipulations. The Cg1, PrL, IL, CA1, CA3, and DG were a downregulation in the c-Fos expression via the cue manipulations. Notably, the Cg1 and NAc were significant differences in the c-Fos expression under the interaction between EE and cue.

3.3. qRT-PCR Assessment for the c-Fos mRNA Expression. To test the c-Fos mRNA expression of EE, cue, and EE×cue in the mPFC, NAc, amygdala, and hippocampus, a 2 × 2 two-way ANOVA was conducted after testing freezing behavior. The results showed that EE manipulations were significant increases in the mPFC ($F(1, 12) = 4.68$, $p < 0.05$) and NAc ($F(1, 12) = 14.01$, $p < 0.05$); however, there was significant decreases in the amygdala ($F(1, 12) = 10.98$, $p < 0.05$) compared to the no EE manipulations. There were nonsignificant differences between the EE and no EE manipulations in the hippocampus ($F(1, 12) = 0.26$, $p > 0.05$). The cue manipulation was significant decreases in the mPFC ($F(1, 12) = 14.01$, $p < 0.05$) and the hippocampus ($F(1, 12) = 16.76$, $p < 0.05$) compared to the no EE manipulation. The cue showed nonsignificant differences in the NAc ($F(1, 12) = 2.52$, $p > 0.05$) and the amygdala ($F(1, 12) = 1.59$, $p > 0.05$). The interaction of EE and cure reveled the nonsignificant differences in the mPFC ($F(1, 12) = 1.95$, $p > 0.05$), the NAc ($F(1, 12) = 1.82$, $p > 0.05$), the amygdala ($F(1, 12) = 1.89$, $p > 0.05$), and the hippocampus ($F(1, 12) = 0.27$, $p > 0.05$; Table 3).

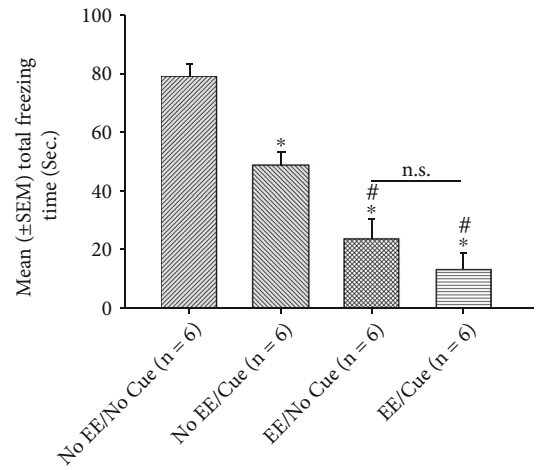

Figure 6: Mean (± SEM) total freezing time from the merged data in sessions 1-3 for the no EE/no cue, no EE/cue, EE/no cue, and EE/cue groups ($n = 6$, per group). EE: environmental enrichment. $^{*}p < 0.05$ indicates significant differences compared to the no EE/no cue group; $\#p < 0.05$ indicates significant differences compared to the no EE/cue group. (n.s.): nonsignificant differences.

Furthermore, one-way ANOVA analysis was conducted to analyze the c-Fos mRNA expression in the mPFC, NAc, amygdala, and hippocampus among the no EE/no cue, no EE/cue, EE/no cue, and EE/cue groups. For the mPFC, the factor of group was significant differences ($F(3, 12) = 6.88$, $p < 0.05$). The post hoc Tukey tests indicated that the c-Fos mRNA expression of the EE/no cue group was significant increases than that of the no EE/no cue group ($p < 0.05$); moreover, the c-Fos mRNA expression of the EE/cue group was significantly decreased compared to the EE/no cue group ($p < 0.05$; Figure 13(a)). The result indicated that the EE manipulation is likely to enhance the c-Fos mRNA expression; however, the cue conduction might decrease the c-Fos mRNA expression in the mPFC.

To test the c-Fos mRNA expression of the NAc, one-way ANOVA analysis showed that significant differences occurred in the factor of the group ($F(3, 12) = 5.85$, $p < 0.05$). The post hoc Tukey test showed that the c-Fos mRNA expression of the EE/no cue group was significantly increased compared to that of the no EE/no cue group ($p < 0.05$; Figure 13(b)), indicating that EE manipulations increased the c-Fos mRNA expression in the NAc.

One-way ANOVA was performed to test the amygdala's c-Fos mRNA expression in the EE and cue manipulations.

Table 2: Effects of environmental enrichment and cue for PTSD in the c-Fos expression for selected brain areas in the situational reminder phase.

	Cg1	PrL	IL	NAc	BLA	CA1	CA3	DG
Environmental enrichment	↑	—	↑	↑	↓	—	—	—
Cue	↓	↓	↓	—	—	↓	↓	↓
Interaction of Cue and environmental enrichment	+	—	—	+	—	—	—	—

Note: (↑): increases; (↓): decreases; (-): nonsignificant difference; (+): $p < 0.05$.

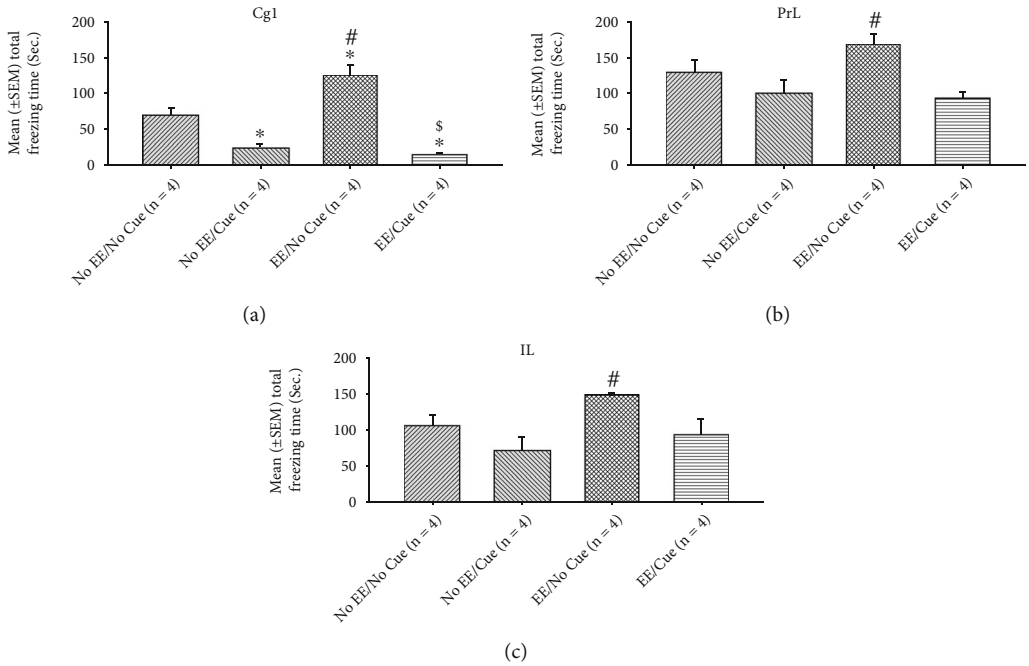

Figure 7: Mean (± SEM) c-Fos expression in the Cg1 (a), PrL (b), and IL (c) for the no EE/no cue ($n = 6$), no EE/cue ($n = 6$), EE/no cue ($n = 6$), and EE/cue groups ($n = 6$). EE: environmental enrichment. $^{*}p < 0.05$ indicates significant differences compared to the no EE/no cue group; $\#p < 0.05$ indicates significant differences compared to the no EE/cue group.

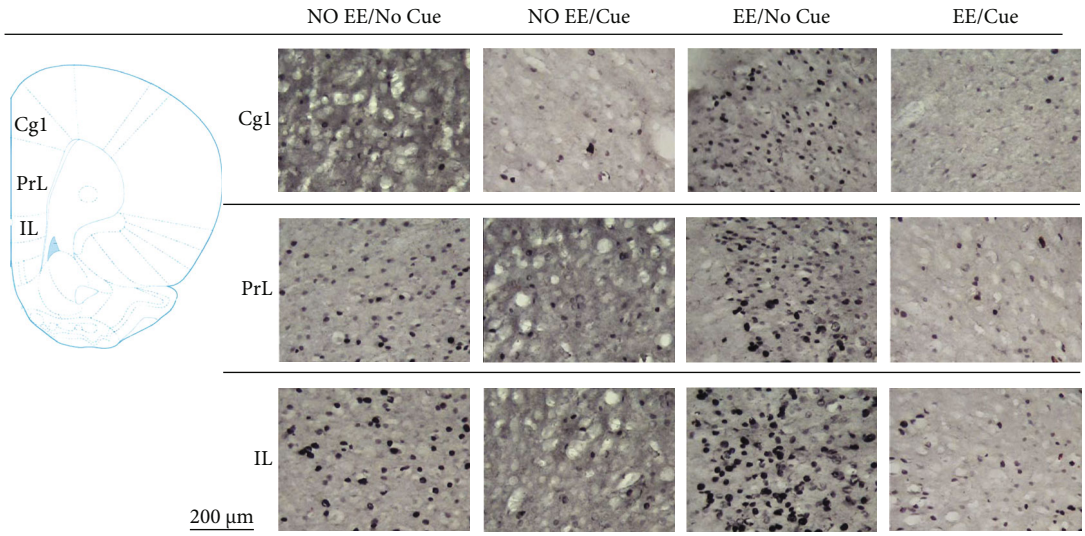

Figure 8: (a) A schematic brain atlas for the Cg1, PrL, and IL of the mPFC. (b) Representative photomicrographs of the c-Fos expression for the Cg1, PrL, and IL in the no EE/no cue, no EE/cue, EE/no cue, and EE/cue groups ($n = 4$, per group). Scale bar represents 200 μm.

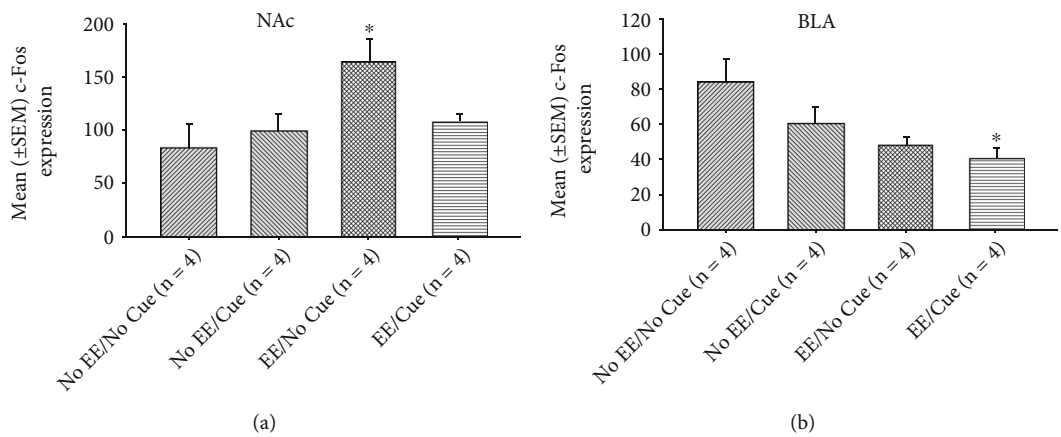

Figure 9: Mean (± SEM) c-Fos expression in the NAc (a) and BLA (b) for the no EE/no cue ($n = 6$), no EE/cue ($n = 6$), EE/no cue ($n = 6$), and EE/cue groups ($n = 6$). EE: environmental enrichment. $^{*}p < 0.05$ indicates significant differences compared to the no EE/no cue group; $\#p < 0.05$ indicates significant differences compared to the no EE/cue group.

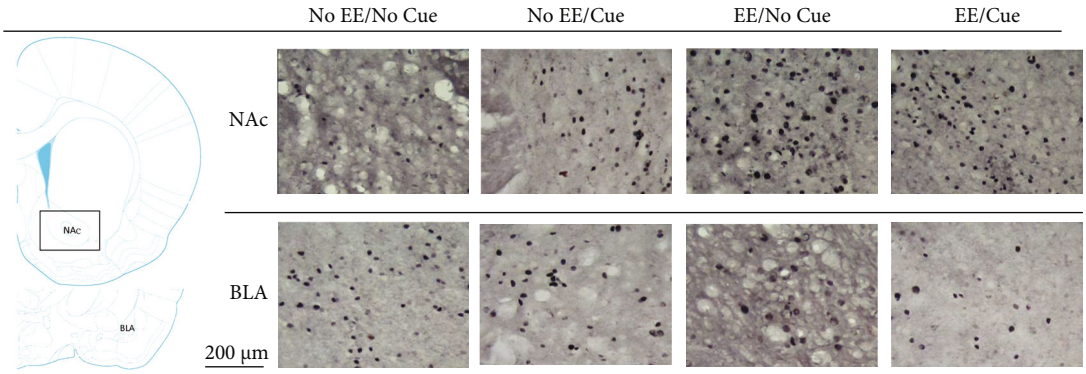

Figure 10: (a) A schematic brain atlas for the NAc and the amygdala's BLA. (b) Representative photomicrographs of the c-Fos expression for the NAc and the amygdala's BLA in the no EE/no cue, no EE/cue, EE/no cue, and EE/cue groups ($n = 4$, per group). Scale bar represents 200 μm.

The results showed that the factor of group had significant differences ($F(3, 12) = 4.82$, $p < 0.05$). The post hoc Tukey tests indicated significant decreases in the EE/no cue and the EE/cue groups compared to the no EE/no cue group ($p < 0.05$; Figure 13(c)), indicating that the EE manipulation decreased the c-Fos mRNA expression in the amygdala.

To test c-Fos mRNA of the hippocampus in the EE and cue manipulations, one-way ANOVA analysis was conducted to show that only the EE/cue group was significant decreases in the c-Fos mRNA expression compared to the no EE/no cue and the EE/no cue groups ($p < 0.05$), respectively (Figure 13(d)). Under cue manipulation, the c-Fos mRNA expression of the hippocampus was significantly decreased.

In conclusion, the results of the c-Fos mRNA expression were similar to the findings of the c-Fos protein expression in the mPFC, NAc, amygdala, and the hippocampus (see Tables 2 and 3).

4. Discussion

The EE manipulations can reduce footshock-induced fear behavior, and the cue manipulation also reduces fear behavior in PTSD symptoms. The present data showed that the EE is better than the cue manipulation to ameliorate fear behavior in PTSD. The combination of EE and cue induced the most significant reduction effect in fear behavior. Cue manipulation is likely a novel treatment to reduce PTSD symptoms, in particular, to fear behavior.

The c-Fos expression data showed that the Cg1, IL, and NAc upregulated the c-Fos expression in fear behavior; however, the BLA downregulated the c-Fos expression in fear behavior for PTSD symptoms through the EE manipulations, indicating the Cg1, IL, NAc, and BLA controlled the EE-induced fear reduction. In contrast, the Cg1, PrL, IL, CA1, CA3, and DG were downregulated in the c-Fos expression after the cue manipulations in fear behavior, indicating the Cg1, PrL, IL, CA1, CA3, and DG contributed to a cue-induced fear reduction in PTSD. The EE and cue had significant interactions in the Cg1 and NAc for the c-Fos expression. Accordingly, the Cg1 and NAc were involved in the interaction of EE and cue.

The data of the c-Fos mRNA expression were similar to the findings of the c-Fos protein expression. EE manipulations upregulated the c-Fos mRNA expression in the mPFC and the NAc; however, it downregulated the c-Fos mRNA

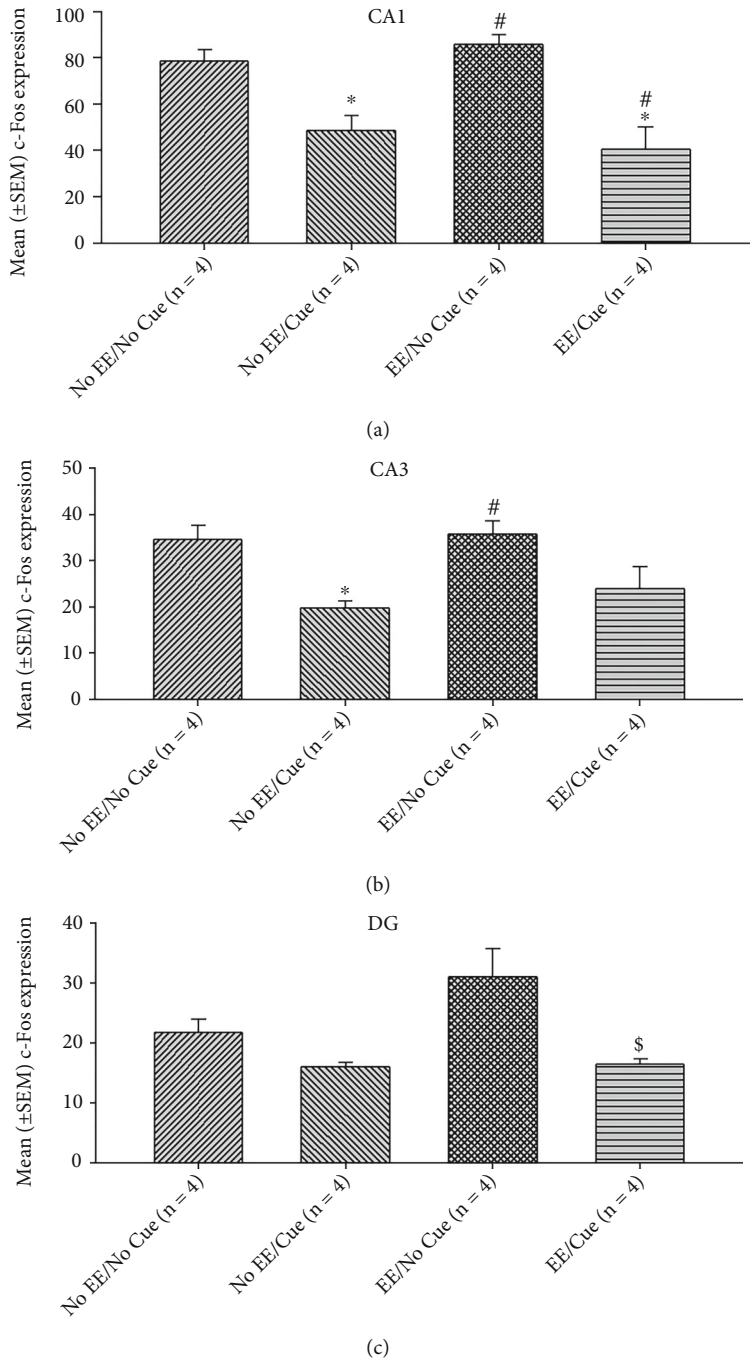

Figure 11: Mean (± SEM) c-Fos expression in the CA1 (a), CA3 (b), and DG (B) for the no EE/no cue ($n = 6$), no EE/cue ($n = 6$), EE/no cue ($n = 6$), and EE/cue groups ($n = 6$). EE: environmental enrichment. $^{*}p < 0.05$ indicates significant differences compared to the no EE/no cue group; $\#p < 0.05$ indicates significant differences compared to the no EE/cue group.

expression in the amygdala. Cue manipulations downregulated the c-Fos mRNA expression in the mPFC and hippocampus. EE and cue interactions did not affect c-Fos mRNA expression in the mPFC, NAc, amygdala, and hippocampus.

4.1. Environmental Enrichment, Cue, and Combination of Environmental Enrichment and Cue to Ameliorate Footshock-Induced Fear Behavior in PTSD Symptoms. In the present data, the EE manipulations ameliorated footshock-induced fear behavior. Therefore, the present data of EE is consistent with the previous findings [33]. Dependent on the previous data, a growing body of evidence showed that the EE manipulations decreased fear- and stress-related behaviors in different levels [6–11, 34]. For example, the behavioral test of EE showed that long-term EE suppressed neonatal isolation-induced contextual freezing, but this type of EE did not change single prolonged

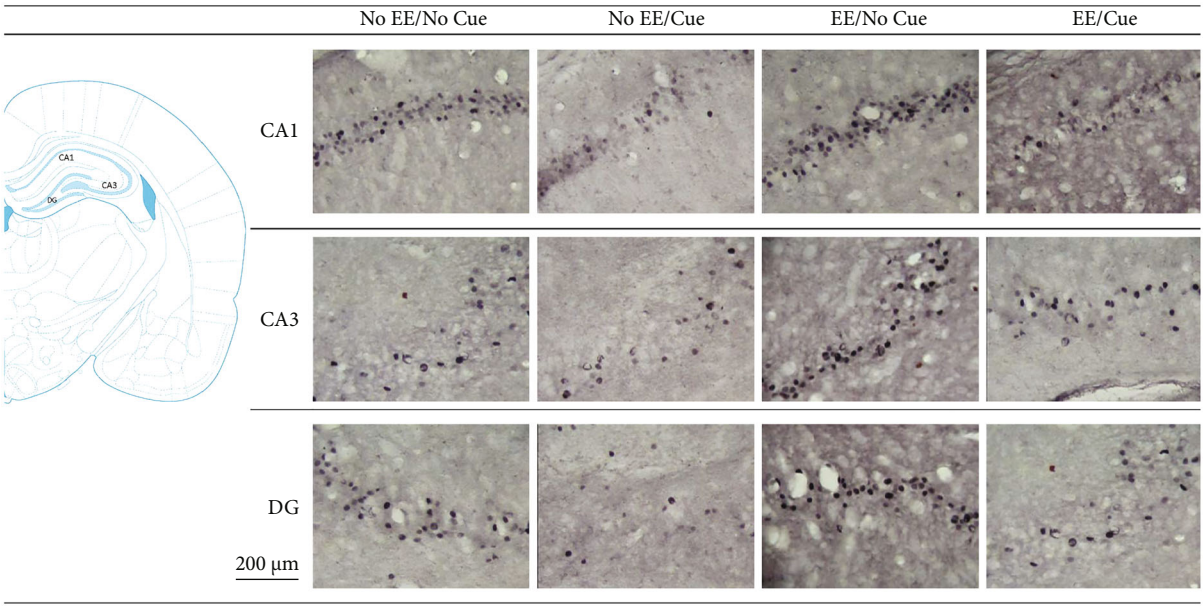

Figure 12: (a) A schematic brain atlas for the Cg1, CA3, and DG of the hippocampus. (b) Representative photomicrographs of the c-Fos expression for the CA1, CA3, and DG of the hippocampus in the no EE/no cue, no EE/cue, EE/no cue, and EE/cue groups ($n = 4$, per group). Scale bar represents 200 μm.

Table 3: Effects of environmental enrichment and cue for PTSD in the normalized c-Fos mRNA expression for selected brain areas in the situational reminder phase.

	mPFC	NAc	Amygdala	Hippocampus
Environmental enrichment	↑	↑	↓	—
Cue	↓	—	—	↓
Interaction of Cue and environmental enrichment	—	—	—	—

Note: (↑): increases; (↓): decreases; (-): nonsignificant difference; (+): $p < 0.05$.

stress related to anxiety behavior or analgesia. Moreover, EE exposures showed to reduce deep-brain stimulation-induced resistant depression and PTSD in the animal model [6]. Alternatively, the examinations of the brain system in EE ameliorations showed that EE could facilitate the negative feedback regulation of the HPA axis for stress rats, and it ameliorated the abnormal behaviors via downregulation of glucocorticoid receptor expression in the hippocampus and hypothalamus [7]. In another study, activations of serotonin and neuropeptide systems through EE manipulations were shown to reduce anxiety behaviors in the PTSD animal model [11]. Regarding the examination of EE manipulations in neuronal levels, animals with EE exposures increased hippocampal cell proliferation and recovered normal behaviors in PTSD symptoms [34]. Additionally, short-term EE exposures potentiated the extinction process of fear via regulating neuropeptide Y-Y1 receptors in the hippocampus [10]. A previous study has demonstrated that EE exposures ameliorated the extended volumes of the hippocampus and central amygdala caused by traumatic stress [8]. Therefore, the EE manipulations may be effectively nonpharmacological interventions in reducing footshock-induced stress or anxiety in behavior, brain systems, and neuronal levels.

On the other hand, manipulating the cue stimulus is likely a novel approach to examine the amelioration of footshock-induced fear behavior. The present results showed that the cue exposure showed a significant decrease in fear behavior induced by footshock; however, this suppressed effect by the cue stimulus was not higher than that of the EE manipulation. The single cue stimulus may cause the different effects between EE and cue, but the EE is composed of a variety of stimuli, including sensory, physical, social, and cognitive components [12, 13]. Therefore, the EE manipulation is similar to the real contextual stimulus.

4.2. Cue and Environmental Enrichment: An Occasion Setter or a CS Associated with the US? These inconsistent data for the EE and cue manipulations should be explained by that the novelty and salient property of stimulus from the EE exposures might be the crucial factor to ameliorate footshock-induced fear behavior [35, 36]. However, the cue stimulus is short of comprehensive and complete stimuli; instead, the cue exposure only has a single stimulus dimension, causing a weaker novelty and salient property to reduce footshock-induced fear behavior. Interestingly, the combination of EE and cue revealed the highest reduction in footshock-induced fear behavior. What reason is the combination of EE and cue due to the highest reduction for footshock-induced fear behavior? It might be two possibilities. One possible reason is that the cue stimulus is served

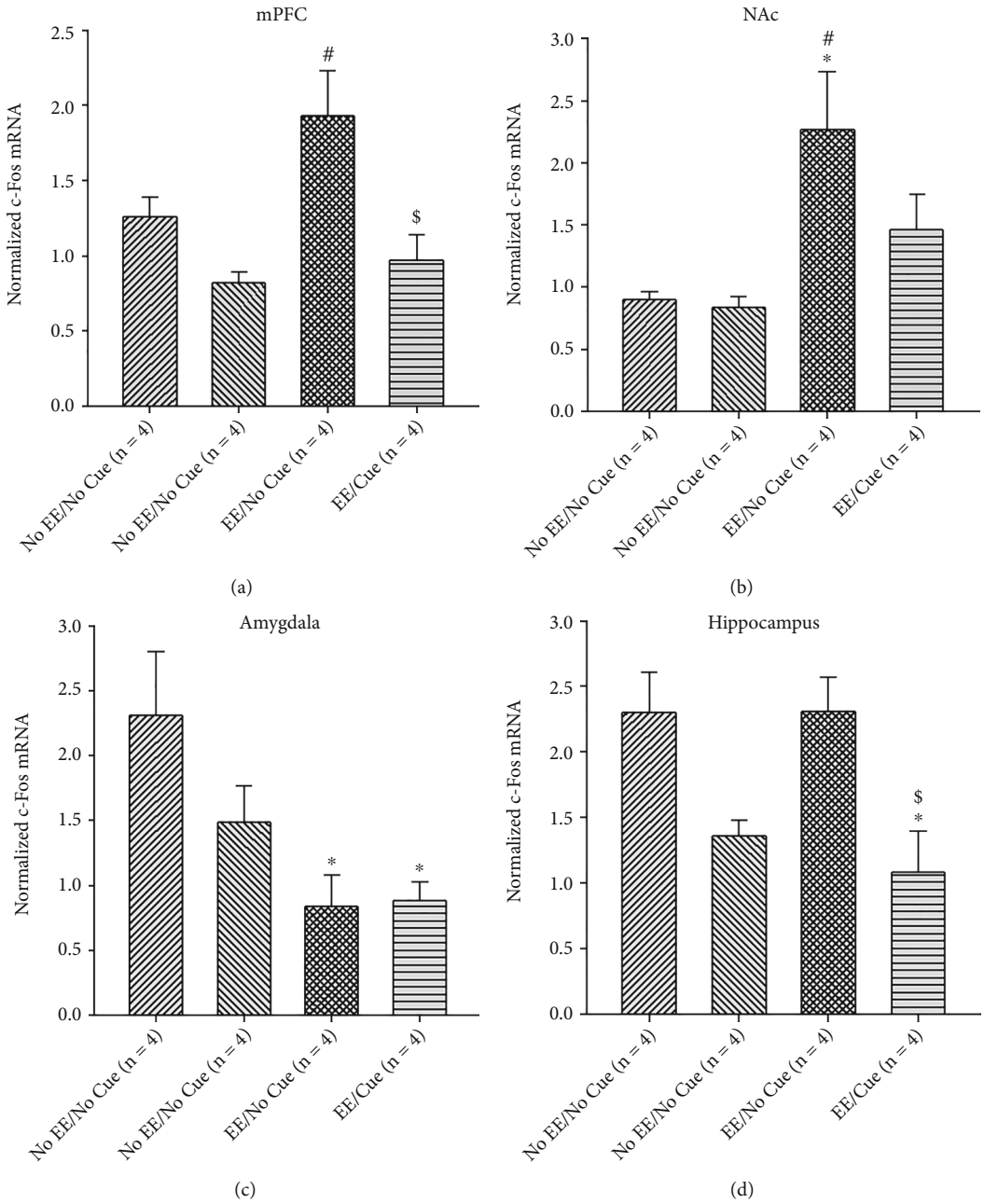

Figure 13: Mean (± SEM) normalized c-Fos mRNA in the mPFC (a), NAc (b), amygdala (c), and hippocampus (d) for the no EE/no cue ($n = 4$), no EE/cue ($n = 4$), EE/no cue ($n = 4$), and EE/cue groups ($n = 4$). EE: environmental enrichment. $^*p < 0.05$ indicates significant differences compared to the no EE/no cue group; $\#p < 0.05$ indicates significant differences compared to the no EE/cue group. $\$p < 0.05$ indicates significant differences compared to the EE/no cue group.

as a conditioned stimulus (CS) to interact with the unconditioned stimulus (US) to modulate the strengths of the conditioned response (CR) by the rule of classical conditioning [37]. Another possible reason is that the cue stimulus is an occasion setter to enhance the CR, fear behavior. Based on the occasion setting theory, the cue stimulus itself was not involved in the association with the US; however, the occasion setter stimulus only modulates the strength of the CR [38, 39]. According to the present study, the cue stimulus seemingly contributed to the association with the US. Therefore, whether the cue stimulus is an occasion setter or a CS remains scrutinized in further studies.

4.3. Involvements of the mPFC, Amygdala, Hippocampus, and NAc in Environmental Enrichment, Cue, or the Combination of Environmental Enrichment and Cue in PTSD Fear Symptoms. To date, no research comprehensively examined how the mPFC, amygdala, and hippocampus contributed to suppressing EE or cue in PTSD symptoms and behaviors. However, amount of research has demonstrated

that the mPFC's neuronal activity inhibited the amygdala's negative emotional responses, and the activation of the amygdala conveyed negative feedback information with the negative value to transmit into the mPFC, which explained the valences of this information [40]. This neural pathway of the mPFC projecting to the amygdala has been shown to regulate PTSD symptoms [41, 42]; moreover, the present data showed that the mPFC-amygdala pathway was seemingly involved in the suppression of EE or cue to PTSD symptoms. On the other hand, the hippocampus was suggested to modulate spatial learning and configural memory [19, 43], and the hippocampus is essential to process memory contents in the process of consolidation from the short-term memory to the long-term memory [44, 45]. Accordingly, the hippocampus was acquired by the amygdala's information to modulate the formation of the configural memory and spatial learning. This result is likely consistent with the recent findings that the amygdala interacted with the hippocampus to mediate emotional learning and memory and PTSD symptoms [46]. The amygdala's pathway with the hippocampus might be another crucial neural pathway for PTSD symptoms.

In the reduced effect of the EE in PTSD fear behavior, it was shown that the Cg1 and IL (but not the PrL) of the mPFC were increased in the c-Fos expression, and it indicated that after chronic EE exposures, the mPFC's executive and inhibitory functions interfered with the activity of the amygdala's BLA, which presented decreases in the c-Fos expression. Decreased c-Fos expression of the BLA indicated that the BLA inhibited the negative emotional responses following the EE exposures.

Alternatively, the present data showed that the c-Fos expression of the NAc significantly increased, indicating that EE exposures might induce dopamine neurotransmitters secretions in the NAc due to the novelty or saliency effects following EE exposures [36, 47, 48]. This novelty and saliency of EE's property may be the critical point that the EE manipulations could reduce the symptoms of psychiatric disorders (e.g., PTSD and depression) or neurological diseases (Parkinson's disease or Alzheimer's disease) [35]. How did the novelty and saliency of EE affect the symptoms of psychiatric and neurological diseases which remain to be investigated further?

In particular, the cue stimulus manipulations showed different results that the Cg1, PrL, and IL of the mPFC and the CA1, CA3, and DG of the hippocampus decreased the c-Fos expression compared to the EE manipulations. Accordingly, the cue manipulations may play a different role in the reduction of footshock-induced fear behavior when compared to the EE manipulations. The cue stimulus is not contextual, and it is likely an occasion setting to modulate fear behavior induced by footshock in the PTSD animal model [39, 49]. Thus, the occasion setter role of the cue fully decreased the c-Fos expression in the whole subregions of the mPFC and the hippocampus. The results indicated that the cue stimulus interfered with the executive and inhibitory functions resulted in the mPFC dysfunctions; moreover, the cue stimulus decreased the c-Fos expression in the CA1, CA3, and DG of the hippocampus, indicating the hippocampus revealed dysfunctions in spatial learning and configural memory. Accordingly, the c-Fos data of the cue stimulus manipulations revealed a worse reduction to footshock-induced fear behavior than those of the EE manipulation and the combination of the EE and cue manipulations.

In the examinations of the interaction between EE and cue, the results showed that significant differences occurred in the Cg1 of the mPFC and the NAc to reduce footshock-induced fear behavior. Why did the c-Fos expression of the Cg1 and NAc produce an interaction between the EE and cue manipulations? It is due to the property of the Cg1 governing the stress or anxiety behaviors and the property of the NAc involving the reward or reinforcement process. This emerged issue should be concerned with further studies.

5. Conclusion

The EE and cue suppressed footshock-induced fear behavior; however, the EE manipulation produced a more potent suppression in footshock-induced fear behavior compared to the cue manipulation; although, the cue stimulus also reduced footshock-induced fear behavior. These data supported the previous findings [6, 8, 11, 35, 36]. For example, EE exposures reduced traumatic stress and increased the volume of the hippocampus and central amygdala [8]; moreover, it ameliorated anxiety [11], depression [6], PTSD symptoms [6], and footshock-induced fear behaviors [35, 36] in the animal model. Notably, the combination of EE and cue manipulations produced the strongest reduction in footshock-induced fear behavior. Under the EE manipulations, c-Fos upregulation occurred in the Cg1, IL, and NAc, but c-Fos downregulation occurred in the BLA. Under cue manipulations, c-Fos downregulation occurred in the Cg1, PrL, IL, CA1, CA3, and DG. The data of the c-Fos mRNA expression were similar with the data of the c-Fos protein expression. The c-Fos mRNA expression was upregulation in the mPFC and amygdala in the EE manipulations; the amygdala was downregulated for the c-Fos mRNA expression in the EE manipulations. Cue manipulations were decreases in the c-Fos mRNA expression for the mPFC and hippocampus. The present findings are likely to offer contributions for novel and nonpharmacological treatments to PTSD symptoms. The present data might help understand the amelioration mechanisms of PTSD fear symptoms in the brain.

Data Availability

The raw data can be accessed through the following link: https://www.dropbox.com/sh/r11cj1scrvggykz/AAC__MvtjwTIMAAQYb9p92p0a?dl=0.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

YHY contributed to the conceptualization, methodology, validation, investigation, data curation, and project administration. YSL contributed to the project administration. CYO contributed to the project administration. KCC contributed to the data curation. FCC contributed to the project administration. ACT contributed to the statistic analysis supports. ACWH contributed to the conceptualization, methodology, formal analysis, investigation, writing-review and editing, and funding acquisition.

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References

[1] H. Cohen, N. Kozlovsky, C. Alona, M. A. Matar, and Z. Joseph, "Animal model for PTSD: from clinical concept to translational research," *Neuropharmacology*, vol. 62, pp. 715–724, 2012.

[2] C. Grillon, S. M. Southwick, and D. S. Charney, "The psychobiological basis of posttraumatic stress disorder," *Molecular Psychiatry*, vol. 1, pp. 278–297, 1996.

[3] D. Kozaric-Kovacic, "Psychopharmacotherapy of posttraumatic stress disorder," *Croatian Medical Journal*, vol. 49, pp. 459–475, 2008.

[4] A. Siegmund and C. T. Wotjak, "Hyperarousal does not depend on trauma-related contextual memory in an animal model of posttraumatic stress disorder," *Physiology & Behavior*, vol. 90, pp. 103–107, 2007.

[5] E. I. Flandreau and M. Toth, "Animal models of PTSD: a critical review," *Current Topics in Behavioral Neurosciences*, vol. 38, pp. 47–68, 2018.

[6] Y. Bhaskar, L. W. Lim, and R. Mitra, "Enriched environment facilitates anxiolytic efficacy driven by deep-brain stimulation of medial prefrontal cortex," *Frontiers in Behavioral Neuroscience*, vol. 12, p. 204, 2018.

[7] Z. Fan, J. Chen, L. Li et al., "Environmental enrichment modulates HPA axis reprogramming in adult male rats exposed to early adolescent stress," *Neuroscience Research*, vol. 172, pp. 63–72, 2021.

[8] Y. Golub, S. F. Kaltwasser, C. P. Mauch et al., "Reduced hippocampus volume in the mouse model of posttraumatic stress disorder," *Journal of Psychiatric Research*, vol. 45, pp. 650–659, 2011.

[9] A. Imanaka, S. Morinobu, S. Toki, and S. Yamawaki, "Importance of early environment in the development of posttraumatic stress disorder-like behaviors," *Behavioural Brain Research*, vol. 173, pp. 129–137, 2006.

[10] G. Lach, M. A. Bicca, A. A. Hoeller, E. C. Santos, A. P. Costa, and T. C. de Lima, "Short-term enriched environment exposure facilitates fear extinction in adult rats: the NPY-Y1 receptor modulation," *Neuropeptides*, vol. 55, pp. 73–78, 2016.

[11] D. Ragu Varman and K. E. Rajan, "Environmental enrichment reduces anxiety by differentially activating serotonergic and neuropeptide Y (NPY)-ergic system in Indian field mouse (Mus booduga): an animal model of post-traumatic stress disorder," *PLoS One*, vol. 10, article e0127945, 2015.

[12] J. C. Brenes, M. Lackinger, G. U. Hoglinger, G. Schratt, R. K. Schwarting, and M. Wohr, "Differential effects of social and physical environmental enrichment on brain plasticity, cognition, and ultrasonic communication in rats," *The Journal of Comparative Neurology*, vol. 524, no. 8, pp. 1586–1607, 2016.

[13] J. Nithianantharajah and A. J. Hannan, "Enriched environments, experience-dependent plasticity and disorders of the nervous system," *Nature Reviews. Neuroscience*, vol. 7, pp. 697–709, 2006.

[14] P. Sampedro-Piquero and A. Begega, "Environmental enrichment as a positive behavioral intervention across the lifespan," *Current Neuropharmacology*, vol. 15, pp. 459–470, 2017.

[15] D. T. Acheson, J. E. Gresack, and V. B. Risbrough, "Hippocampal dysfunction effects on context memory: possible etiology for posttraumatic stress disorder," *Neuropharmacology*, vol. 62, pp. 674–685, 2012.

[16] N. Kaouane, E. G. Ducourneau, A. Marighetto, M. Segal, and A. Desmedt, "False opposing fear memories are produced as a function of the hippocampal sector where glucocorticoid receptors are activated," *Frontiers in Behavioral Neuroscience*, vol. 14, p. 144, 2020.

[17] F. Steiger, F. Nees, M. Wicking, S. Lang, and H. Flor, "Behavioral and central correlates of contextual fear learning and contextual modulation of cued fear in posttraumatic stress disorder," *International Journal of Psychophysiology*, vol. 98, pp. 584–593, 2015.

[18] N. C. Huff and J. W. Rudy, "The amygdala modulates hippocampus-dependent context memory formation and stores cue-shock associations," *Behavioral Neuroscience*, vol. 118, pp. 53–62, 2004.

[19] L. E. Jarrard, "On the role of the hippocampus in learning and memory in the rat," *Behavioral and Neural Biology*, vol. 60, pp. 9–26, 1993.

[20] S. Tanimoto, T. Nakagawa, Y. Yamauchi, M. Minami, and M. Satoh, "Differential contributions of the basolateral and central nuclei of the amygdala in the negative affective component of chemical somatic and visceral pains in rats," *The European Journal of Neuroscience*, vol. 18, pp. 2343–2350, 2003.

[21] J. D. Bremner, "Traumatic stress: effects on the brain," *Dialogues in Clinical Neuroscience*, vol. 8, no. 4, pp. 445–461, 2006.

[22] S. H. Chang, Y. H. Yu, A. He, C. Y. Ou, B. C. Shyu, and A. C. W. Huang, "BDNF protein and BDNF mRNA expression of the medial prefrontal cortex, amygdala, and hippocampus during situational reminder in the PTSD animal model," *Behavioural Neurology*, vol. 2021, Article ID 6657716, 13 pages, 2021.

[23] B. M. Elzinga and J. D. Bremner, "Are the neural substrates of memory the final common pathway in posttraumatic stress disorder (PTSD)?," *Journal of Affective Disorders*, vol. 70, pp. 1–17, 2002.

[24] L. M. Shin, S. L. Rauch, and R. K. Pitman, "Amygdala, medial prefrontal cortex, and hippocampal function in PTSD," *Annals of the New York Academy of Sciences*, vol. 1071, pp. 67–79, 2006.

[25] M. M. Miller and B. S. McEwen, "Establishing an agenda for translational research on PTSD," *Annals of the New York Academy of Sciences*, vol. 1071, no. 1, pp. 294–312, 2006.

[26] N. Korem, R. Lange, C. J. Hillard, and I. Akirav, "Role of beta-catenin and endocannabinoids in the nucleus accumbens in

extinction in rats exposed to shock and reminders," *Neuroscience*, vol. 357, pp. 285–294, 2017.

[27] C. Piao, X. Deng, X. Wang, Y. Yuan, Z. Liu, and J. Liang, "Altered function in medial prefrontal cortex and nucleus accumbens links to stress-induced behavioral inflexibility," *Behavioural Brain Research*, vol. 317, pp. 16–26, 2017.

[28] J. C. Kung, T. C. Chen, B. C. Shyu, S. Hsiao, and A. C. Huang, "Anxiety- and depressive-like responses and c-fos activity in preproenkephalin knockout mice: oversensitivity hypothesis of enkephalin deficit-induced posttraumatic stress disorder," *Journal of Biomedical Science*, vol. 17, no. 1, p. 29, 2010.

[29] Y. H. Yu, C. Y. Ou, B. C. Shyu, and A. C. W. Huang, "Basolateral amygdala but not medial prefrontal cortex contributes to chronic fluoxetine treatments for PTSD symptoms in mice," *Behavioural Neurology*, vol. 2020, Article ID 8875087, 8 pages, 2020.

[30] T. Takahashi, K. Shimizu, K. Shimazaki, H. Toda, and M. Nibuya, "Environmental enrichment enhances autophagy signaling in the rat hippocampus," *Brain Research*, vol. 1592, pp. 113–123, 2014.

[31] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, "NIH image to ImageJ: 25 years of image analysis," *Nature Methods*, vol. 9, pp. 671–675, 2012.

[32] T. D. Schmittgen, "Real-time quantitative PCR," *Methods*, vol. 25, pp. 383–385, 2001.

[33] H. Hendriksen, B. Olivier, and R. S. Oosting, "From non-pharmacological treatments for post-traumatic stress disorder to novel therapeutic targets," *European Journal of Pharmacology*, vol. 732, pp. 139–158, 2014.

[34] H. Hendriksen, J. Prins, B. Olivier, and R. S. Oosting, "Environmental enrichment induces behavioral recovery and enhanced hippocampal cell proliferation in an antidepressant-resistant animal model for PTSD," *PLoS One*, vol. 5, article e11943, 2010.

[35] A. J. Hannan, "Environmental enrichment and brain repair: harnessing the therapeutic effects of cognitive stimulation and physical activity to enhance experience-dependent plasticity," *Neuropathology and Applied Neurobiology*, vol. 40, no. 1, pp. 13–25, 2014.

[36] C. Girbovan and H. Plamondon, "Environmental enrichment in female rodents: considerations in the effects on behavior and biochemical markers," *Behavioural Brain Research*, vol. 253, pp. 178–190, 2013.

[37] I. P. Pavlov, *Conditioned reflexes*, I. P. Pavlov, Ed., Oxford University Press, Oxford, UK, 1927.

[38] K. M. Fraser and P. C. Holland, "Occasion setting," *Behavioral Neuroscience*, vol. 133, pp. 145–175, 2019.

[39] N. A. Schmajuk, J. A. Lamoureux, and P. C. Holland, "Occasion setting: a neural network approach," *Psychological Review*, vol. 105, pp. 3–32, 1998.

[40] M. J. Kim, R. A. Loucks, A. L. Palmer et al., "The structural and functional connectivity of the amygdala: from normal emotion to pathological anxiety," *Behavioural Brain Research*, vol. 223, pp. 403–410, 2011.

[41] T. F. Giustino and S. Maren, "The role of the medial prefrontal cortex in the conditioning and extinction of fear," *Frontiers in Behavioral Neuroscience*, vol. 9, p. 298, 2015.

[42] M. Koenigs and J. Grafman, "Posttraumatic stress disorder: the role of medial prefrontal cortex and amygdala," *The Neuroscientist*, vol. 15, pp. 540–548, 2009.

[43] J. W. Rudy and R. J. Sutherland, "Configural association theory and the hippocampal formation: an appraisal and reconfiguration," *Hippocampus*, vol. 5, pp. 375–389, 1995.

[44] I. Izquierdo, C. R. Furini, and J. C. Myskiw, "Fear memory," *Physiological Reviews*, vol. 96, no. 2, pp. 695–750, 2016.

[45] S. Languille, S. Davis, P. Richer, C. Alcacer, S. Laroche, and B. Hars, "Extracellular signal-regulated kinase activation is required for consolidation and reconsolidation of memory at an early stage of ontogenesis," *The European Journal of Neuroscience*, vol. 30, no. 10, pp. 1923–1930, 2009.

[46] A. J. McDonald and D. D. Mott, "Functional neuroanatomy of amygdalohippocampal interconnections and their role in learning and memory," *Journal of Neuroscience Research*, vol. 95, pp. 797–820, 2017.

[47] A. Badiani, M. M. Oates, H. E. W. Day, S. J. Watson, H. Akil, and T. E. Robinson, "Amphetamine-induced behavior, dopamine release, and c-fos mRNA expression: modulation by environmental novelty," *The Journal of Neuroscienc.*, vol. 18, no. 24, pp. 10579–10593, 1998.

[48] A. Badiani, M. M. Oates, H. E. W. Day, S. J. Watson, H. Akil, and T. E. Robinson, "Environmental modulation of amphetamine-induced c-fos expression in D1 versus D2 striatal neurons," *Behavioural Brain Research*, vol. 103, pp. 203–209, 1999.

[49] P. Holland, "Ocassion setting in Pavlovian conditioning," in *The Psychology of Learning and Motivation*, D. L. Medin, Ed., pp. 69–125, CA: Academic Press, San Diego, 1992.

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Research Article

Solitary Nitric Oxide Signaling Mediates Mild Stress-Induced Anxiety and Norepinephrine Release in the Bed Nucleus of the Stria Terminalis during Protracted Ethanol Withdrawal

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Ethanol withdrawal (EtOHW) alters the pattern of neurohormonal and behavioral response toward internal and external stimuli, which mediates relapse to alcohol use even after a long period of abstinence. Increased noradrenergic signaling from the nucleus tractus solitarius (NTS) to the bed nucleus of the stria terminalis (BNST) during EtOHW underlies withdrawal-induced anxiety, while nitric oxide synthase (NOS) inhibitors injected into the periaqueductal area attenuate EtOHW-induced anxiety. Therefore, this study investigated the involvement of NOS within the NTS in anxiety and increased norepinephrine (NE) release in the BNST during protracted EtOHW in rats exposed to a mild stress. Rats were intraperitoneally administered 3 g/kg/day EtOH for 21 days followed by 28 days of withdrawal, and on the 28th day of withdrawal, the rats were subjected to restraint stress for 7 minutes. The elevated plus maze test was employed to evaluate anxiety-like behavior in rats, and in vivo microdialysis was used to measure the extracellular NE level in the BNST. In elevated plus maze tests, EtOHW rats but not EtOH-naive rats exhibited anxiety-like behavior when challenged with 7-minute mild restraint stress, which was, respectively, mitigated by prior intra-NTS infusion of the nitric oxide scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), nonselective NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME), or selective neuronal NOS (nNOS) inhibitor 7-nitroindazole (7-NI). Each of these agents also decreased the plasma corticosterone levels in EtOHW rats. In in vivo microdialysis, prior intra-NTS infusion of carboxy-PTIO, L-NAME, or 7-NI attenuated the mild stress-induced NE release in the BNST of EtOHW rats. Additionally, EtOHW rats showed increased solitary nNOS gene and protein expression. Moreover, the anxiolytic effect of intra-NTS administration of 7-NI was abolished by subsequent intra-NTS administration of sodium nitroprusside. These results suggest that elevation of solitary nitric oxide signaling derived from nNOS mediates stress-precipitated anxiety and norepinephrine release in the BNST during protracted EtOHW.

1. Introduction

Relapse is a major barrier in alcoholism therapy [1]. Ethanol withdrawal (EtOHW) leads to adaptation of neurotransmission in the brain that often persists long after complete remission of EtOHW symptoms. This adaptation sensitizes physiological and behavioral responses to internal and external stimuli, characterized by exacerbated pathophysiological responses toward mild stress or even nonstress stimuli [2]. This phenomenon during EtOHW is a stress sensitization that mimics the allodynia in pain medicine in which innocuous stimuli provoke a painful sensation, resulting in relapse even after a long period of abstinence [3].

Elevated anxiety during EtOHW is the major negative emotional component for alcoholism relapse [4], and several lines of evidence indicate that a heightened norepinephrine (NE) signaling in the bed nucleus of the stria terminalis (BNST) is responsible for it. The BNST is a component of the extended amygdala; NE signaling in the BNST along with corticotrophin releasing factor (CRF) is the key factor

mediating the anxiety associated with withdrawal of drugs of abuse [5]. NE triggers the extracellular release of CRF, and EtOHW increases the extracellular NE level in the central nucleus of the amygdala (another component of the extended amygdala) [6] and the CRF level in the BNST [7], which underlie EtOHW-induced anxiety. EtOH and morphine share many pharmacological similarities including induction of extracellular NE release in the BNST [8], and extracellular NE release is increased in the BNST in both spontaneous and naloxone-precipitated morphine withdrawal [9, 10]. Therefore, the extracellular NE level may be enhanced in the BNST during EtOHW in association with elevated anxiety. Moreover, Valdez et al. [11] reported that acute mild-restraint stress (AMRS) produced significant anxiety in rats during protracted EtOHW but not in their EtOH-naive counterparts, indicating an allodynia-like phenomenon. Taken together, these observations suggest that AMRS may induce sensitized NE release in the BNST, which is associated with anxiety in rats during protracted EtOHW.

The BNST is a complex consisting of multiple nuclei, broadly divided into anterior and posterior, dorsal and ventral, and medial and lateral parts, and it is heavily innervated by noradrenergic projections arising from both A1/A2 in the nucleus tractus solitarius (NTS) and A6 (locus coeruleus) [12]. However, immunocytochemical and retrograde tracer analyses indicated that the noradrenergic inputs to the ventral BNST (vBNST) are derived mainly from the NTS-A2 [13, 14] and are critical for the negative emotions induced by withdrawal of drugs of abuse. For example, opiate withdrawal precipitated by opioid receptor antagonists was shown to increase NE release in the vBNST and to enhance c-Fos expression in both the vBNST and vBNST-projecting NTS-A2 neurons, whereas intra-vBNST infusion of β-adrenergic antagonists reduced the withdrawal-associated conditioned place aversion [14–16]. Neurochemical lesions of the noradrenergic bundle originating from the NTS-A2, but not from A6, attenuated the withdrawal aversion [14]. These observations suggest increased activity in the NTS-vBNST noradrenergic pathway during withdrawal of drugs of abuse, which is attributed to activation of NTS-A2 neurons. A wide range of neurotransmitters and neuropeptides, including glutamate, gamma-aminobutyric acid (GABA), nitric oxide (NO), galanin, and neuropeptide Y, are present in the NTS-A2 and regulate NE neuronal activities [17]. It is well documented that NO synthase (NOS) inhibiters have anxiolytic effects [18–20]; moreover, the NO system plays an important role in EtOHW anxiety and NE release induced by drug abuse. Bonassoli et al. [21] reported that EtOHW activated NO-producing neurons in the brainstem, while local infusion of NOS inhibitors into the brainstem regions such as the periaqueductal gray matter area and the dorsal raphe nucleus mitigates EtOHW anxiety [22, 23]. Our previous studies indicated that acute nicotine induces NE release in the hypothalamus and amygdala via the NTS NO pathway [24, 25]. These findings suggest that the NTS NO system may mediate the enhanced NTS-vBNST noradrenergic activities during protracted EtOHW.

Taken together, all the above observations lead to speculation that there may be abnormally heightened NTS-vBNST NE neuronal activities mediated by the NTS NO system during protracted EtOHW, which underlie anxiety in response to challenge with otherwise innocuous stimuli. To test this, in the present study, rats were exposed to a 7-minute AMRS during protracted EtOHW, and the effects of intra-NTS infusion of NOS inhibitors on anxiety-like behavior and NE release in the vBNST of EtOHW rats were examined.

2. Materials and Methods

2.1. Animals and Surgery. Eight-week-old male Sprague–Dawley rats (250-270 g) were provided by the Laboratory Animal Center at Qiqihar Medical University (Qiqihar, China) and housed in individual cages under standardized conditions (12 : 12 hour light/dark cycle, 21–23°C, free access to food and water). All rats were randomly assigned to the various experimental groups. All experimental procedures were approved by the Animal Care and Use Committee of Qiqihar Medical University (approval number: QMUAECC-2016-28) and were performed adhering to the National Institutes of Health guidelines.

After 7 days of acclimatization, the rats were stereotactically (Kopf Instruments, Tujunga, CA) implanted with bilateral microinjection guide cannulae targeting the NTS and/or a unilateral microdialysis probe guide cannula targeting the right ventral vBNST under sodium pentobarbital (50 mg/kg, intraperitoneally) anesthesia. In brief, after fixing the rat on the instrument, a longitudinal midline incision was made on the shaved and disinfected scalp, creating the surgical window by exposing the bregma and the lambda and clearing the skull surface using sterile cotton-tips dipped in 3% H_2O_2 solution. The coordinates of the bregma and the lambda were used to level the skull and act as the references, and the hydrogen peroxide solution (also a disinfectant) was used to eliminate the soft tissues to prevent a possible infection afterward. After the confirmation of a horizontally levelled skull, three small holes were drilled on the skull, respectively, targeting the right ventral vBNST and both sides of the NTS according to their coordinates, and guide cannulae were implanted through the holes and secured with two small screws and dental cement. The coordinates of the NTS and vBNST relative to bregma, according to the atlas of Paxinos and Watson [26], were as follows: anterior–posterior, −13.6 and−0.3 mm; medial–lateral, 0.8 and 1.4 mm; and dorsal ventral, 6.5 and 7.5 mm, respectively. The microinjection guide cannulae were positioned 1.5 mm above the targets. During the surgery, a small amount of lubricant eye ointment was applied to each rat to prevent corneal damage, and antibiotics and pain killers were also used for the postsurgery care. At the end of each experiment, the rats were euthanized and decapitated, the brain was extracted and stored at -80°C, and then coronal cryotome sections were made to observe the probe and injection positions, and if more accurate histological confirmation was desired, a Coomassie blue staining was used; finally, the rats with the correct targets were included for statistical analysis.

2.2. Experimental Protocols and Collection of Blood and NTS Tissues. After a 7-day recovery period after the surgery, the

rats were intraperitoneally administered 3 g/kg/day EtOH (20% w/v) or saline for 21 days followed by 28 days of withdrawal. On the 28th day of EtOHW or saline withdrawal, the rats received 7-minute AMRS in a 6.4-cm-diameter plastic cylinder constructed from a 500 mL mineral water bottle and underwent in vivo microdialysis or a behavioral test in an elevated plus maze (EPM) (Figure 1).

Immediately after the EPM test, the rats were euthanized and decapitated. Trunk blood was collected to measure plasma corticosterone (CORT) levels. The entire brain was removed and stored at −80°C, and NTS tissues were obtained by the punch-out technique [25] according to the coordinates outlined above for Western blot analysis.

2.3. Intra-NTS Microinfusion. To examine the possible effects of NOS inhibitors on AMRS-induced anxiety and NE release in the vBNST during protracted EtOHW, bilateral intra-NTS microinfusion of the following agents was performed: NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO; 0.2 nmol/100 nL each side; Sigma-Aldrich, St. Louis, MO), nonselective NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME; 30 nmol/100 nL each side; Sigma-Aldrich), selective neuronal NOS (nNOS) inhibitor 7-nitroindazole (7-NI; 30 nmol/100 nL each side; Sigma-Aldrich), selective endothelial NOS (eNOS) inhibitor L-N5-(1-iminoethyl)ornithine dihydrochloride (L-NIO; 3 nmol/100 nL each side, Sigma-Aldrich), and NO donor sodium nitroprusside (SNP; 0.1 nmol/100 nL each side; Sigma-Aldrich). The agents were infused using a 30-gauge injector (1.5 mm longer than the guide cannula) through the intra-NTS guide cannulae (22-gauge) using a microinjection pump for 45 seconds. All drugs were dissolved in modified Ringer's solution containing 150 mM NaCl, 3.0 mM KCl, 1.4 mM $CaCl_2$, and 0.8 mM $MgCl_2$ in 10 mM phosphate buffer, pH 7.2. All drugs were introduced 5 minutes before AMRS except for SNP, which was given after intra-NTS administration of 7-NI, immediately after AMRS, to examine the effect of subsequent intra-NTS administration of SNP on the anxiolytic action of 7-NI. The rats were subjected to the EPM test 5 minutes after AMRS (Figure 1).

2.4. EPM Test. On day 28 of EtOHW, after treatment with drugs and exposure to AMRS, the rats were examined in the EPM to measure anxiety-like behaviors as described previously [6]. Briefly, the EPM was composed of two closed arms (50 cm long × 10 cm wide, with black walls 40 cm high) and two open arms (without walls), which were arranged perpendicularly, elevated above the ground and video recorded using a video tracking system (Shanghai Xinruan Technology Co., Shanghai, China). During the examination, each rat was placed in the center of the EPM, and the number of entries into the arms and the time spent in each arm were monitored for 5 minutes. The percentages of number of entries into the open arms and time spent in the open arms, relative to the total entries/time, were calculated as follows: $\%\text{Entries}_{\text{into open arms}} = \text{Entries}_{\text{into open arms}}/(\text{Entries}_{\text{into open arms}} + \text{Entries}_{\text{into closed arms}}) \times 100\%$ and $\%\text{Time}_{\text{spent in open arms}} = \text{Time}_{\text{spent in open arms}}/(\text{Time}_{\text{spent in open arms}} + \text{Time}_{\text{spent in closed arms}}) \times 100\%$.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA). Blood samples (1 mL from each rat) collected in chilled microcentrifuge tubes containing 20 μL EDTA (20 mg/mL) were centrifuged for 10 minutes at 1,500 × g and 4°C to separate the plasma. Plasma CORT levels were determined using a commercial ELISA kit (Abcam, Cambridge, UK) in accordance with the manufacturer's instructions, and values are presented as nanograms per milliliter.

2.6. Measurement of Extracellular NE. On day 28 of EtOHW, a microdialysis probe (CMA11, 2-mm membrane length, 6,000 Da; Carnegie Medicine, Stockholm, Sweden) was inserted into the vBNST via the guide cannula. The probe was constantly perfused (1.5 μL/min) with Krebs–Ringer buffer (147 mM NaCl, 3.4 mM $CaCl_2$, and 4.0 mM KCl in polished water) containing 5 μM nomifensine using a microinjection pump for 2 hours. Next, microdialysates were collected at 15-minute intervals in microcentrifuge tubes containing 1 μL 5% perchloric acid, and basal NE levels were determined by measuring three consecutive dialysates prior to experimental administration. NE levels in the microdialysates were measured by injecting a 15 μL dialysate into a high-performance liquid chromatography system equipped with a coulometric detector (Coulochem II; ESA Laboratories, Bedford, MA) [25]. After measuring the basal NE level, the rats received bilateral intra-NTS injection of carboxy-PTIO, L-NAME, 7-NI, or L-NIO. Five minutes after injection of the drugs, the rats were subjected to AMRS. Microdialysates were continuously collected during these experimental processes and thereafter (60 minutes total) and analyzed for NE levels (Figure 1).

2.7. Western Blot Analysis. The whole NTS tissues from each rat that underwent the designated experimental schedule were homogenized in lysis buffer containing 20 mM Tris, 5 mM EDTA, 1% Nonidet P-40 (v/v), and phosphatase and protease inhibitors. The homogenates were centrifuged for 20 minutes at 16,000 × g and 4°C, and the total protein content in each supernatant was quantified by bicinchoninic acid assay. The proteins were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The proteins in the membrane were reacted with the following primary antibodies: rabbit polyclonal antibodies to nNOS, eNOS, phospho-nNOS Ser1417, β-actin (all from Abcam), and phospho-eNOS Ser1177 (Cell Signaling Technology, Beverly, MA). The membranes were then reacted with a peroxidase-conjugated anti-rabbit secondary antibody (Cell Signaling Technology). Finally, the bands of the proteins of interest were visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ), densitometrically analyzed with the aid of ImageJ, and the relative expressions of the proteins were calculated as follows (the β-actin was used as the loading control): $\%\text{of protein expression relative to the control group} = (\text{density of the designated protein}/\text{Density of } \beta-\text{actin}$

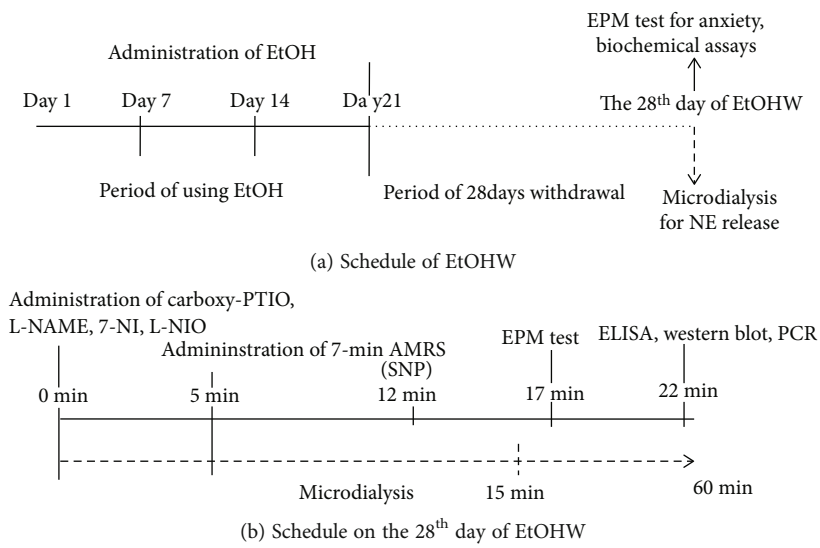

Figure 1: (a) Time schedules for EtOHW and (b) the 28th day of EtOHW.

$)_{\text{treated}}/(\text{density of the designated protein/density of } \beta-\text{actin})_{\text{control}} \times 100\%$.

2.8. Real-Time RT-PCR. Total RNA was extracted from the whole NTS tissues of each rat that underwent the designated experimental schedule using TRIzol Reagent (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA using a reverse-transcription PCR kit (TaKaRa Bio Inc., Shiga, Japan). Real-time PCR amplification was performed on the CFX-96 PCR system (Bio-Rad, Hercules, CA) using SYBR green premix (TaKaRa) in accordance with the manufacturer's protocol. The following primers were synthesized by Beijing Liuhe BGI Gene, Co. (Beijing, China): nNOS, 5′-TCCCTCTAGCCAAAGAATTTCTCG-3′ (forward) and 5′-GGTAGGTGCTGGTGCTTTCAA-3′ (reverse); β-actin, 5-GTCGTACCACTGGCATTGTG-3 (forward) and 5-GCCATCTCTTGCTCGAAGTC-3 (reverse). The housekeeping gene β-actin was used to normalize the gene expression measurements, and the relative level of specific mRNA was calculated using the following formula and presented as $2^{-\Delta\Delta CT}$: $\Delta CT = CT_{nNOS} - CT_{\beta-actin}$, $\Delta\Delta CT = \Delta CT_{EtOH} - \Delta CT_{Saline}$.

2.9. Statistical Analysis. All data are presented as the mean ± SEM. Two-way analysis of variance (ANOVA) with Bonferroni posthoc test, or one-way ANOVA followed by Newman–Keuls post-hoc test was performed to statistically evaluate data. All analyses were performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA), and differences with p values <0.05 were considered statistically significant.

3. Results

3.1. Effects of Carboxy-PTIO, L-NAME, 7-NI, and L-NIO on AMRS-Induced Anxiety during Protracted EtOHW. EtOHW produces anxiety-like behaviors in rats that disappear spontaneously after a certain time depending on a number of variables, including animal species and EtOH regimen. In preliminary experiments, we found that EtOHW rats did not display any anxiety-like behaviors in the EPM test 28 days after the final dose of a 21-day EtOH treatment, in agreement with the observation by Valdez et al. [11]. Meanwhile, 7-minute AMRS alone did not produce any anxiety-like behaviors in EtOH-naive rats. Therefore, to observe the interactive effect of EtOHW and an AMRS, in the present study, the rats were exposed to 7-minute AMRS on day 28 of EtOHW and then subjected to the EPM test.

As shown in Figures 2(a) and 2(b) (the data were analyzed by two-way ANOVA followed by Bonferroni post-hoc test), 28 days after the final dose of EtOH, the EtOHW rats did not show any significant anxiety-like behaviors; however, when exposed to 7-minute AMRS, EtOHW rats but not saline-treated controls displayed substantial anxiety-like behaviors, which were manifested by fewer visits and less time spent in the open arms [$\%\text{entries}_{\text{into open arms}}$: $F_{(\text{drug})} = 12.89$, $p < 0.01$, $F_{(\text{stress})} = 12.52$, $p < 0.01$, $F_{(\text{drug}\times\text{stress})} = 8.71$, $p < 0.01$; saline/non-AMRS group ($n = 8$) versus EtOH/AMRS group ($n = 8$), $p < 0.001$; EtOH/non-AMRS group versus EtOH/AMRS group, $p < 0.001$; saline/AMRS group ($n = 8$) versus EtOH/AMRS group, $p < 0.001$; $\%\text{time}_{\text{spent in open arms}}$: $F_{(\text{drug})} = 13.24$, $p < 0.01$, $F_{(\text{stress})} = 18.09$, $p < 0.001$, $F_{(\text{drug}\times\text{stress})} = 15.01$, $p < 0.001$; saline/non-AMRS group versus EtOH/AMRS group, $p < 0.001$; EtOH/non-AMRS group versus EtOH/AMRS group, $p < 0.001$; saline/AMRS group versus EtOH/AMRS group, $p < 0.001$]. However, as also seen in Figures 2(c) and 2(d) (the data analyzed by one-way ANOVA followed by Newman–Keuls posthoc test), intra-NTS infusion of carboxy-PTIO, L-NAME, or 7-NI, but not L-NIO, attenuated the anxiety-like behaviors [$\%\text{entries}_{\text{into open arms}}$: $F_{(5,42)} = 9.22$, $p < 0.001$, EtOH/vehicle/non-AMRS group ($n = 8$) versus EtOH/vehicle/AMRS group ($n = 8$), $p < 0.001$, EtOH/vehicle/AMRS group versus EtOH/carboxy-PTIO/AMRS group ($n = 8$), $p < 0.01$; EtOH/vehicle/AMRS group versus EtOH/

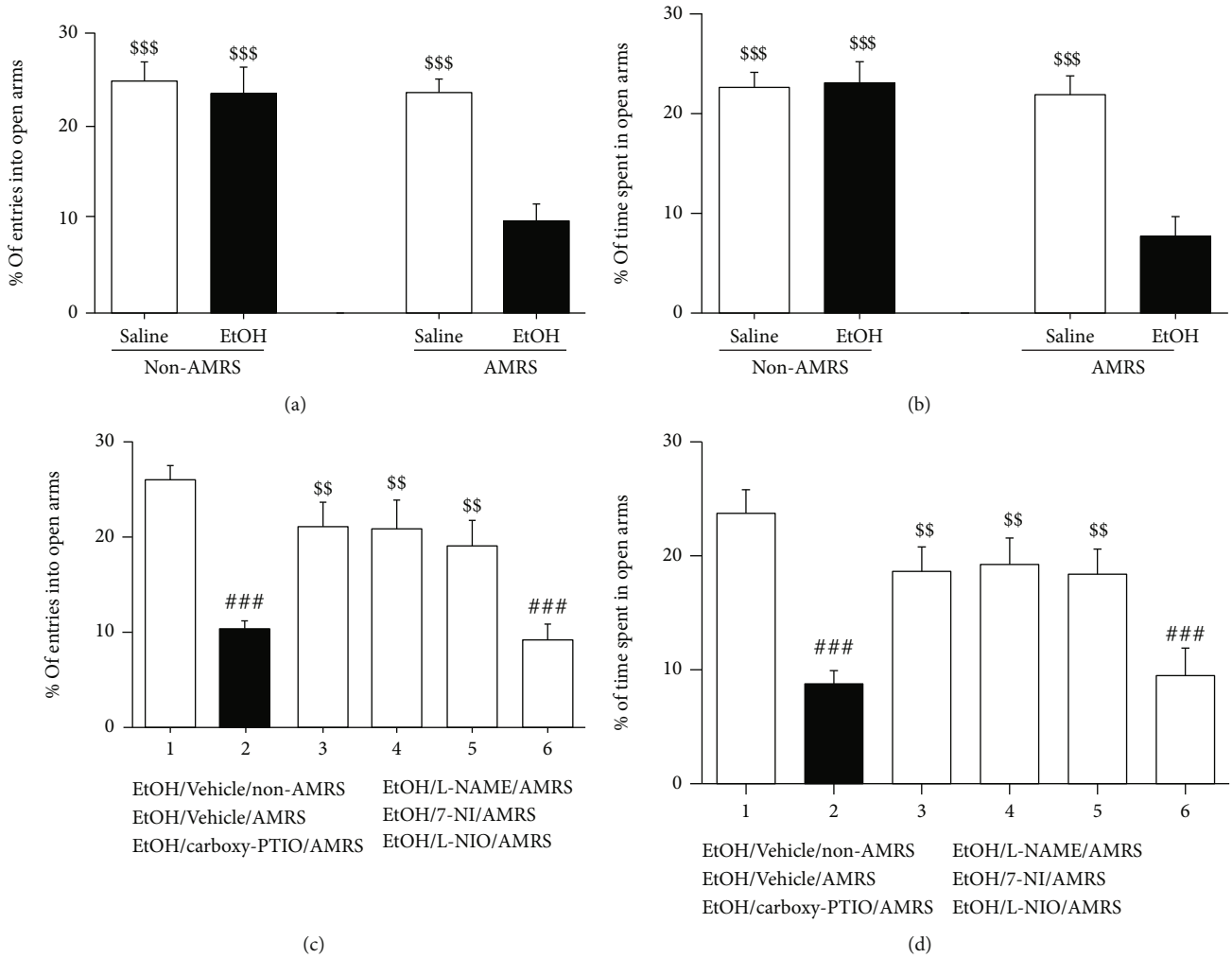

Figure 2: Effects of carboxy-PTIO, L-NAME, 7-NI, and L-NIO on AMRS-induced anxiety-like behavior. Data are expressed as the mean ± SEM ($n = 8$). (a, c) Percentage of numbers of entries into open arms of EPM. (b, d) Percentage of time spent in open arms. $^{###}p < 0.001$ versus saline/vehicle/non-AMRS group; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ versus EtOH/AMRS group or EtOH/vehicle/AMRS group.

L-NAME/AMRS group ($n = 8$), $p < 0.01$; EtOH/vehicle/AMRS group versus EtOH/7-NI/AMRS group ($n = 8$), $p < 0.01$; EtOH/vehicle/AMRS group versus EtOH/L-NIO/AMRS group ($n = 8$), $p > 0.05$; $\%time_{\text{spent in open arms}}$: $F_{(5,42)} = 8.05$, $p < 0.001$, EtOH/vehicle/non-AMRS group versus EtOH/vehicle/AMRS group, $p < 0.001$, EtOH/vehicle/AMRS group versus EtOH/carboxy-PTIO/AMRS group, $p < 0.01$; EtOH/vehicle/AMRS group versus EtOH/L-NAME/AMRS group, $p < 0.01$; EtOH/vehicle/AMRS group versus EtOH/7-NI/AMRS group, $p < 0.01$; EtOH/vehicle/AMRS group versus EtOH/L-NIO/AMRS group, $p > 0.05$]. Moreover, at the same dose, intra-NTS 7-NI alone did not produce any significant behavioral changes in rats (data not shown).

3.2. Effects of Carboxy-PTIO, L-NAME, 7-NI, and L-NIO on AMRS-Induced Plasma CORT Secretion. Consistent with the results of the behavioral, as seen in Figure 3(a), AMRS increased the plasma CORT level in EtOHW rats but not in saline-treated control rats [$F_{(\text{drug})} = 19.92$, $p < 0.001$, $F_{(\text{stress})} = 32.25$, $p < 0.001$, $F_{(\text{drug}\times\text{stress})} = 13.96$, $p < 0.01$; saline/non-AMRS group ($n = 6$) versus EtOH/AMRS group ($n = 6$), $p < 0.001$; EtOH/non-AMRS group ($n = 6$) versus EtOH/AMRS group, $p < 0.001$; saline/AMRS group versus EtOH/AMRS group, $p < 0.001$]. However, as shown in Figure 3(b), intra-NTS infusion of carboxy-PTIO [$F_{(5,30)} = 11.71$, $p < 0.001$; EtOH/vehicle/non-AMRS group ($n = 6$) versus EtOH/vehicle/AMRS group ($n = 6$), $p < 0.001$, EtOH/vehicle/AMRS group versus EtOH/carboxy-PTIO/AMRS group ($n = 6$), $p < 0.01$], L-NAME [EtOH/vehicle/AMRS group versus EtOH/L-NAME/AMRS group ($n = 6$), $p < 0.01$], or 7-NI [EtOH/vehicle/AMRS group versus EtOH/7-NI/AMRS group ($n = 6$), $p < 0.01$], but not L-NIO [EtOH/vehicle/AMRS group versus EtOH/L-NIO/AMRS group ($n = 6$), $p > 0.05$], inhibited the increased CORT secretion.

3.3. Effects of Carboxy-PTIO, L-NAME, 7-NI, and L-NIO on AMRS-Induced NE Release in the vBNST. No significant differences in the basal extracellular NE level in the vBNST were found between the groups [saline/vehicle/non-AMRS group ($n = 7$): 4.25 ± 0.32 (pg/15 uL); saline/vehicle/AMRS

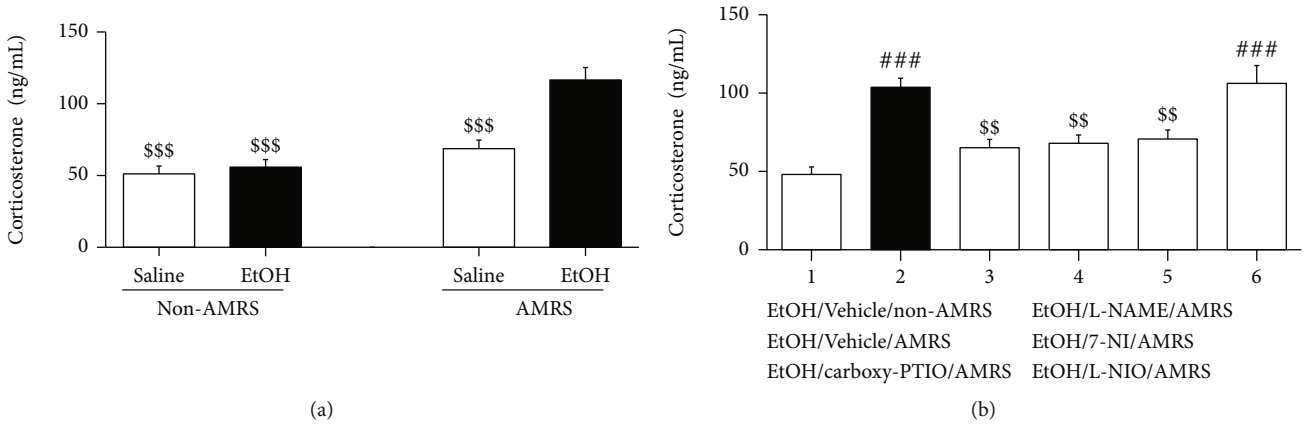

Figure 3: Effects of carboxy-PTIO, L-NAME, 7-NI, and L-NIO on AMRS-induced plasma CORT secretion. Data are expressed as mean ± SEM ($n = 6$). $^{###}p < 0.001$ versus saline/vehicle/non-AMRS group; $^{\$\$\$}p < 0.001$ versus EtOH/AMRS group or EtOH/vehicle/AMRS group.

group ($n = 7$): 4.75 ± 0.48; EtOH/vehicle/non-AMRS group ($n = 7$): 4.64 ± 0.41; EtOH/vehicle/AMRS group ($n = 7$): 5.19 ± 0.44; EtOH/carboxy-PTIO/AMRS group ($n = 7$): 4.38 ± 0.37; EtOH/L-NAME/AMRS group ($n = 7$): 4.23 ± 0.38; EtOH/7-NI/AMRS group ($n = 7$): 5.25 ± 0.37; EtOH/L-NIO/AMRS group ($n = 7$): 4.81 ± 0.37]. As seen in Figures 4(a) and 4(b), two-way ANOVA and Bonferroni posthoc comparisons revealed that 7-minute AMRS significantly increased NE release in EtOHW rats but not in saline-treated control rats [in Figure 4(a), $F_{(\text{treatment})} = 0.07$, $p > 0.05$, $F_{(\text{time})} = 2.25$, $p > 0.05$, $F_{(\text{treatment}\times\text{time})} = 0.70$, $p > 0.05$; in Figure 4(b), $F_{(\text{treatment})} = 16.79$, $p < 0.001$; $F_{(\text{time})} = 25.63$, $p < 0.001$, $F_{(\text{treatment}\times\text{time})} = 9.55$, $p < 0.001$; 15 min: saline/vehicle/AMRS group versus EtOH/vehicle/AMRS group, $p < 0.001$; 30 min saline/vehicle/AMRS group versus EtOH/vehicle/AMRS group, $p < 0.05$]. However, as shown in Figure 4(c) (the data were analyzed by two-way ANOVA followed by Bonferroni posthoc test), intra-NTS infusion of carboxy-PTIO [$F_{(\text{treatment})} = 9.62$, $p < 0.001$, $F_{(\text{time})} = 87.92$, $p < 0.001$, $F_{(\text{treatment}\times\text{time})} = 4.53$, $p < 0.001$; 15 min: EtOH/vehicle/non-AMRS group EtOH/vehicle/AMRS group, $p < 0.001$, EtOH/vehicle/AMRS versus EtOH/carboxy-PTIO/AMRS group, $p < 0.001$; 30 min: EtOH/vehicle/non-AMRS group versus EtOH/vehicle/AMRS group, $p < 0.01$, EtOH/vehicle/AMRS group versus EtOH/carboxy-PTIO/AMRS group, $p < 0.01$], L-NAME [15 min: EtOH/vehicle/AMRS group versus EtOH/L-NAME/AMRS group, $p < 0.001$; 30 min: EtOH/vehicle/AMRS group versus EtOH/L-NAME/AMRS group, $p < 0.05$], or 7-NI [15 min: EtOH/vehicle/AMRS group versus EtOH/7-NI/AMRS group, $p < 0.001$; 30 min: EtOH/vehicle/AMRS group versus EtOH/7-NI/AMRS group, $p < 0.05$], but not L-NIO [15 min: EtOH/vehicle/AMRS group versus EtOH/L-NIO/AMRS group, $p > 0.05$; 30 min: EtOH/vehicle/AMRS group versus EtOH/L-NIO/AMRS group, $p > 0.05$], prevented these increases. In addition, intra-NTS L-NIO alone did not significantly affect NE release in naive rats (data not shown).

3.4. nNOS and eNOS Protein and nNOS mRNA Expressions during Protracted EtOHW. As shown in Figure 5, Western blot analyses revealed the EtOHW significantly increased the protein level of (total) nNOS in the NTS compared with saline treatment [$F_{(\text{drug})} = 93.69$, $p < 0.001$, $F_{(\text{stress})} = 0.04$, $p > 0.05$, $F_{(\text{drug}\times\text{stress})} = 0.08$, $p > 0.05$; saline/non-AMRS group ($n = 5$) versus EtOH/non-AMRS group ($n = 5$), $p < 0.001$; saline/AMRS group ($n = 5$) versus EtOH/AMRS group ($n = 5$), $p < 0.001$], whereas the AMRS did not alter nNOS protein expression (saline/non-AMRS group versus saline/AMRS group, $p > 0.05$; EtOH/non-AMRS group versus EtOH/AMRS group, $p > 0.05$). However, the AMRS markedly enhanced phospho-nNOS levels in EtOHW rats [$F_{(\text{drug})} = 45.06$, $p < 0.001$, $F_{(\text{stress})} = 53.45$, $p < 0.001$, $F_{(\text{drug}\times\text{stress})} = 36.38$, $p < 0.001$; EtOH/AMRS group ($n = 5$) versus EtOH/non-AMRS group ($n = 5$), $p < 0.001$; EtOH/AMRS group versus saline/AMRS group ($n = 5$), $p < 0.001$; EtOH/AMRS group versus saline/non-AMRS group ($n = 5$), $p < 0.001$] but not in saline-treated controls (saline/AMRS group versus saline/non-AMRS group, $p > 0.05$). Additionally, neither EtOHW nor AMRS significantly affected eNOS or phospho-eNOS protein expressions ($p > 0.05$ for all).

In agreement with the Western blot data, as seen in Figure 6, PCR analysis showed enhanced nNOS mRNA expression in the NTS of EtOHW rats compared with saline-treated controls [$F_{(\text{drug})} = 84.40$, $p < 0.001$, $F_{(\text{stress})} = 0.04$, $p > 0.05$, $F_{(\text{drug}\times\text{stress})} = 0.06$, $p > 0.05$; saline/non-AMRS group ($n = 6$) versus EtOH/non-AMRS group ($n = 6$), $p < 0.001$; saline/AMRS group ($n = 6$) versus EtOH/AMRS group ($n = 6$), $p < 0.001$]. The AMRS did not alter nNOS mRNA expression (saline/non-AMRS group versus saline/AMRS group, $p > 0.05$; EtOH/non-AMRS group versus EtOH/AMRS group, $p > 0.05$).

3.5. Effects of Subsequent Intra-NTS Infusion of SNP on the Anxiolytic Action of 7-NI during EtOHW. To further determine the involvement of solitary nNOS in AMRS-induced anxiety during protracted EtOHW, another cohort of EtOHW rats was sequentially treated with 7-NI and SNP and then tested in the EPM (Figure 1). As shown in Figure 7, one-way ANOVA and posthoc tests showed that intra-NTS infusion of 7-NI once again blocked the AMRS-

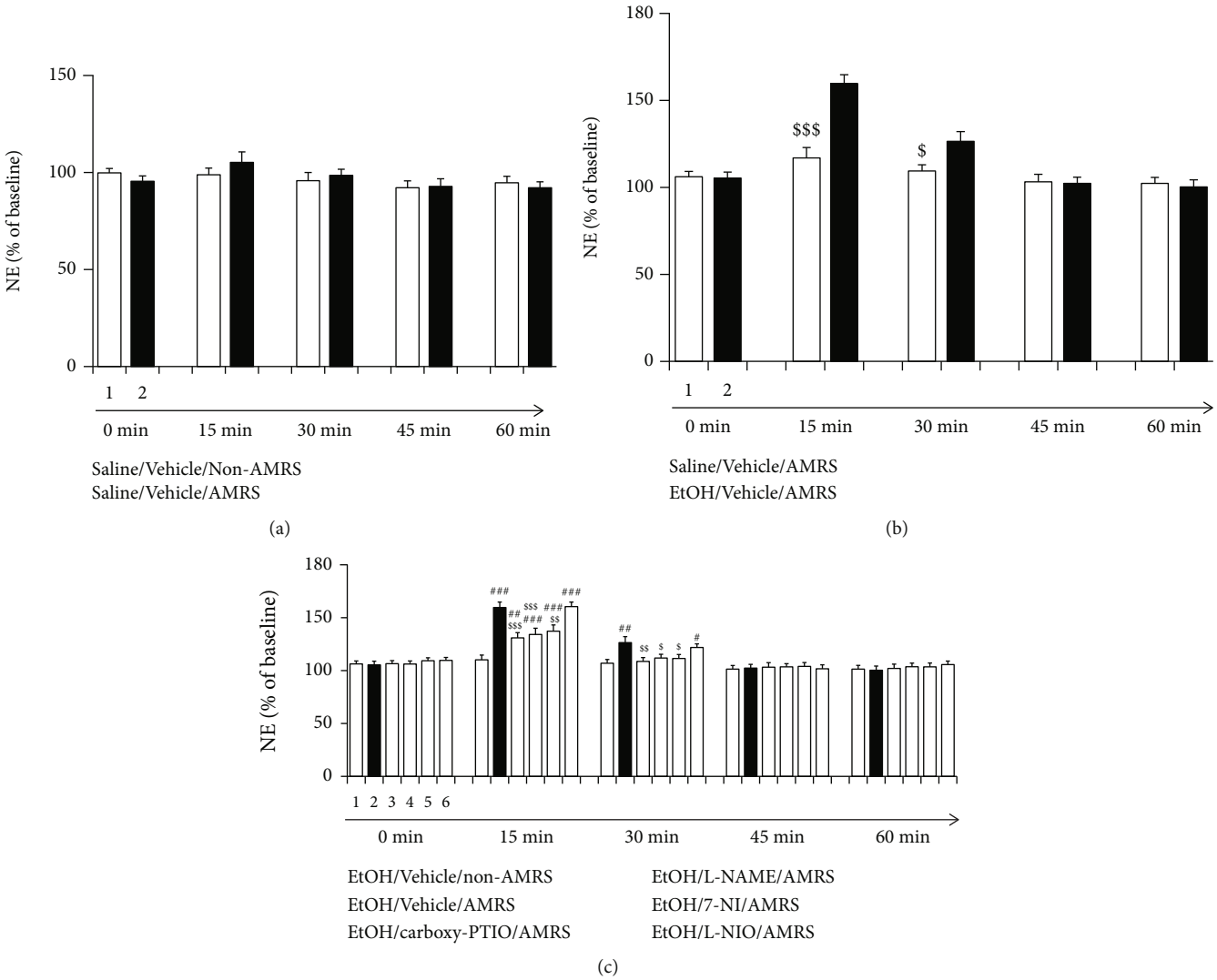

Figure 4: Effects of carboxy-PTIO, L-NAME, 7-NI, and L-NIO on AMRS-induced NE release in the vBNST. Data are expressed as the mean ± SEM ($n = 7$) of the percentage of the baseline. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus EtOH/vehicle/non-AMRS group; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ versus EtOH/vehicle/AMRS group.

induced anxiety during EtOHW; however, subsequent administration of SNP abolished this anxiolytic effect [$\%\text{entries}_{\text{into open arms}}$: $F_{(3,20)} = 15.87$, $p < 0.001$; saline/vehicle/AMRS/vehicle group ($n = 6$) versus EtOH/vehicle/AMRS/vehicle group ($n = 6$), $p < 0.001$; EtOH/vehicle/AMRS/vehicle group versus EtOH/7-NI/AMRS/vehicle group ($n = 6$), $p < 0.001$; EtOH/7-NI/AMRS/vehicle group versus EtOH/7-NI/AMRS/SNP group ($n = 6$), $p < 0.01$; saline/vehicle/AMRS/vehicle group versus EtOH/7-NI/AMRS/SNP group, $p < 0.001$; $\%\text{time}_{\text{spent in open arms}}$: $F_{(3,20)} = 11.17$, $p < 0.001$; saline/vehicle/AMRS/vehicle group versus EtOH/vehicle/AMRS/vehicle group, $p < 0.001$; EtOH/vehicle/AMRS/vehicle group versus EtOH/7-NI/AMRS/vehicle group, $p < 0.01$; EtOH/7-NI/AMRS/vehicle group versus EtOH/7-NI/AMRS/SNP group, $p < 0.05$; saline/vehicle/AMRS/vehicle group versus EtOH/7-NI/AMRS/SNP group, $p < 0.01$].

4. Discussion

The results of the present study showed that 7-minute AMRS provoked anxiety-like behaviors, enhanced plasma CORT secretion, and sensitized NE release in the vBNST in rats treated with EtOH but not saline, at 28 days after the final dose of EtOH or saline. However, all of these behavioral, hormonal, and neurochemical abnormalities were attenuated by prior intra-NTS infusion of carboxy-PTIO, L-NAME, or 7-NI, but not by L-NIO. EtOHW elevated nNOS, but not eNOS, protein expression in the NTS, concomitant with an increased nNOS mRNA level, and the AMRS increased the phosphorylation rate of nNOS in the NTS of EtOHW rats. Moreover, intra-NTS injection of SNP after 7-NI administration abolished the expected anxiolytic action of 7-NI. Taken together, these results suggest a critical role of solitary nNOS in anxiety and vBNST NE

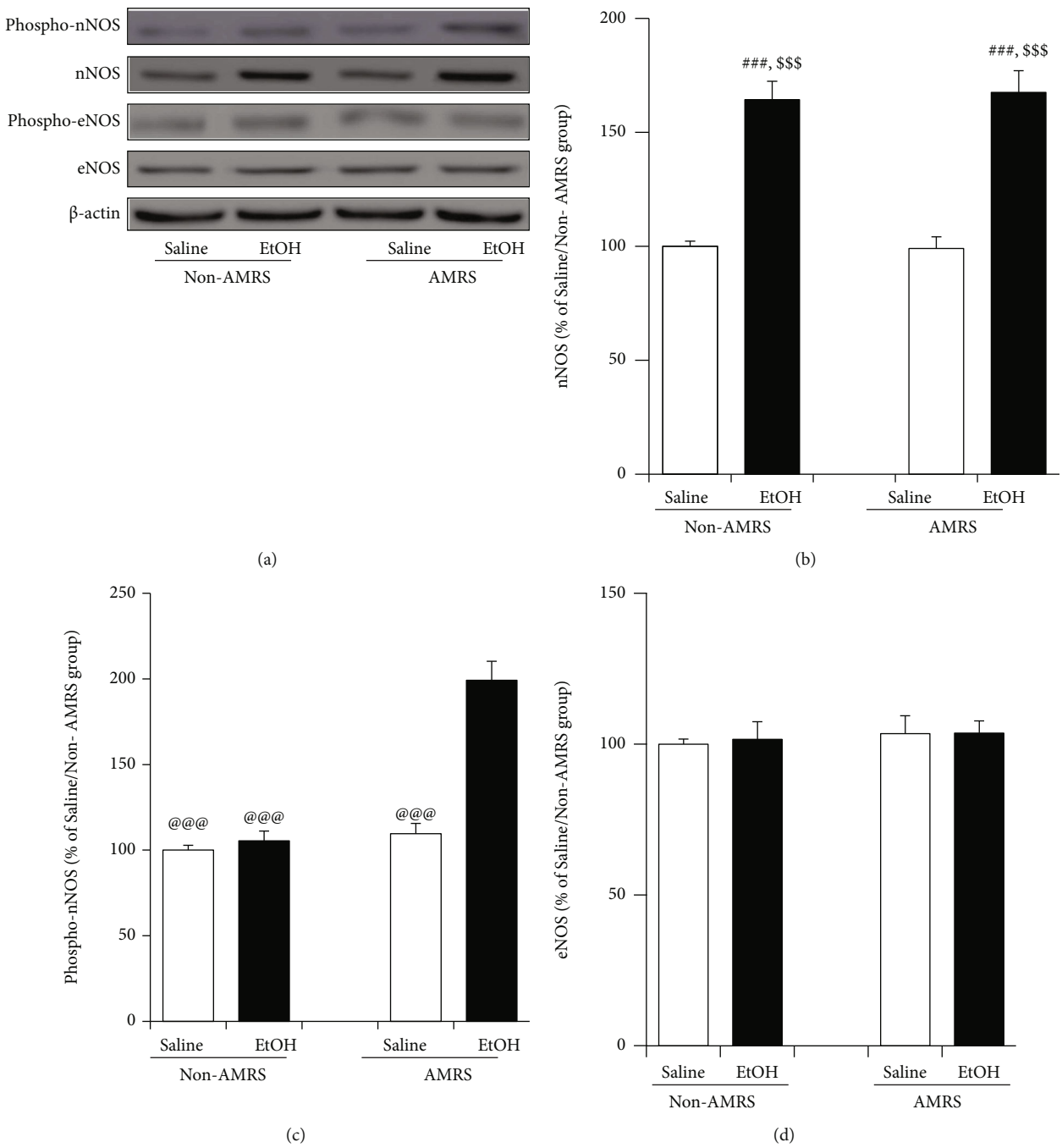

Figure 5: Continued.

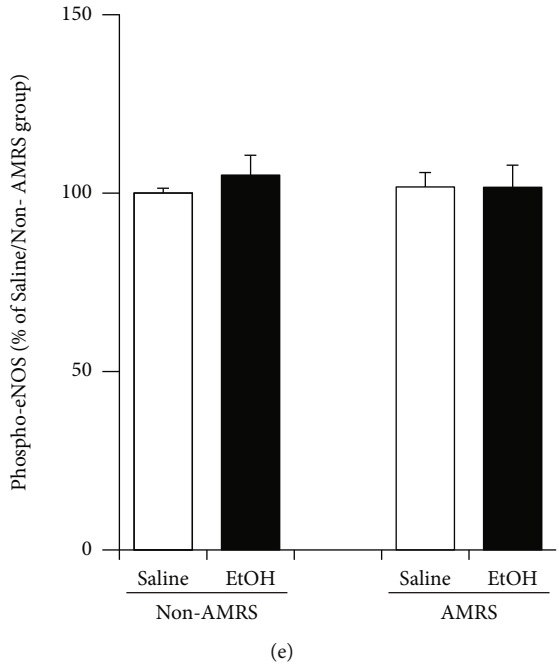

Figure 5: Solitary nNOS and eNOS protein expressions during EtOHW. Data are expressed as mean ± SEM ($n = 5$) of the percentage of saline/non-AMRS group. $^{###}p < 0.001$ versus saline/non-AMRS group; $^{\$\$\$}p < 0.001$ versus saline/AMRS group; $^{@@@}p < 0.001$ versus EtOH/AMRS group.

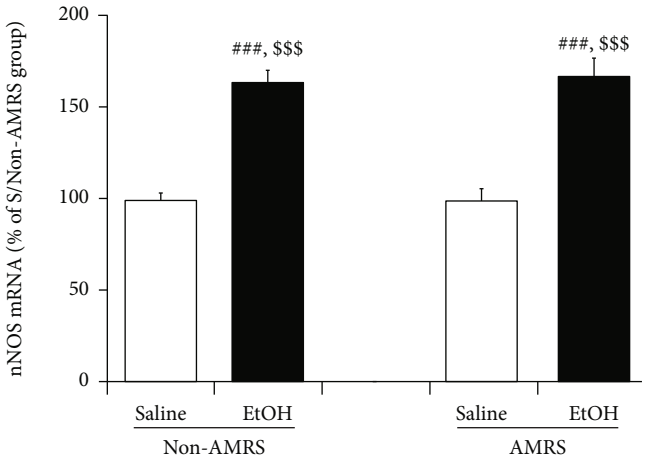

Figure 6: Solitary nNOS mRNA expressions during EtOHW. Data are expressed as mean ± SEM ($n = 6$) of the percentage of saline/non-AMRS group. $^{###}p < 0.001$ versus saline/non-AMRS group; $^{\$\$\$}p < 0.001$ versus saline/AMRS group.

release induced by acute mild stress during protracted EtOHW.

The susceptibility to stress during protracted EtOHW alters neurotransmission responses to certain stimuli that are normally innocuous to provoke pathophysiological consequences [2, 27]. To mimic this, in the present study, rats were subjected to 7-minute AMRS at 28 days after cessation of EtOH when spontaneous EtOHW anxiety-like symptoms had disappeared. Similar to the results from Valdez et al. [11], the AMRS produced anxiety in the EtOHW rats but not in their saline-treated counterparts. These results also were in agreement with the report by Ostroumov et al. [28] indicating that restraint stress increased EtOH self-administration via altered tegmental GABA signaling. However, in the present study, the anxiety was blocked by prior intra-NTS injection of carboxy-PTIO, L-NAME, or 7-NI, but not L-NIO. These results were consistent with the reported anxiolytic effects of NOS inhibitors [18, 19] and compatible with the reports of Bonassoli et al. [22] and Gonzaga et al. [23] revealing that infusion of a nonselective NOS inhibitor, a selective nNOS inhibitor, or a selective inducible NOS (iNOS) inhibitor (N-([3-(aminomethyl)phenyl]methyl) ethanimidamide dihydrochloride) into the dorsolateral periaqueductal gray matter or into the dorsal raphe nucleus, respectively, attenuated EtOHW anxiety in rats 24 or 48 hours after discontinuation of EtOH. The expression of iNOS is induced in response to inflammatory and immune stimuli [29], and chronic EtOH may cause dysregulation of the immune system in the brain that can persist over a certain withdrawal time, while increased production of proinflammatory factors has shown to sensitize EtOHW anxiety [30–32]. However, in the present study, we did not examine the involvement of solitary iNOS, since it was not detected in the NTS of the protracted EtOHW rats in a preliminary experiment. Moreover, in the present study, the anxiolytic effects of the above mentioned agents were hormonally supported because ELISA showed that carboxy-PTIO, L-NAME, and 7-NI, but not L-NIO, attenuated the increase in plasma CORT secretion induced by AMRS during EtOHW. These results collectively suggest that solitary NO signaling via nNOS contributes to the anxiety induced by acute mild stress during protracted EtOHW.

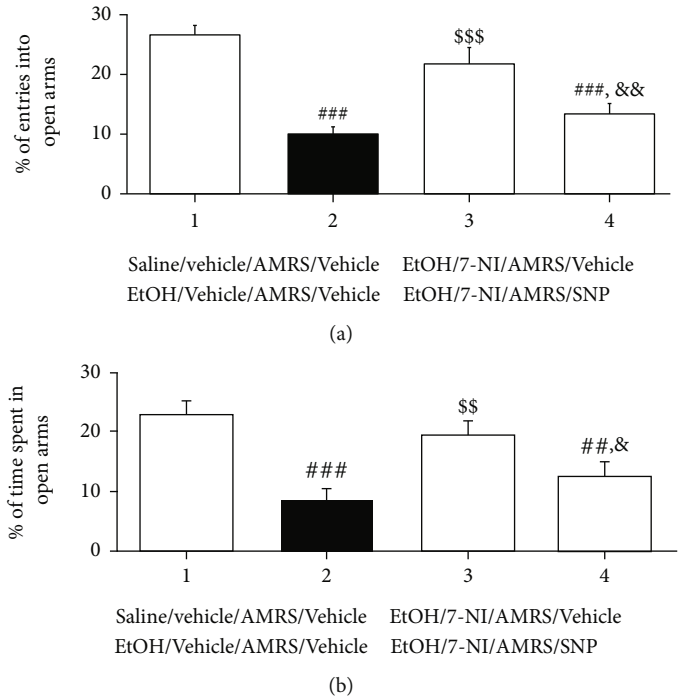

Figure 7: Effects of subsequent intra-NTS infusion of SNP on the anxiolytic action of 7-NI. Data are expressed as the mean ± SEM ($n = 6$). (a) Percentage of numbers of entries into open arms. (b) Percentage of time spent in open arms. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus saline/vehicle/AMRS/vehicle group; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ versus EtOH/vehicle/AMRS/vehicle group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ versus EtOH/7-NI/AMRS/vehicle group.

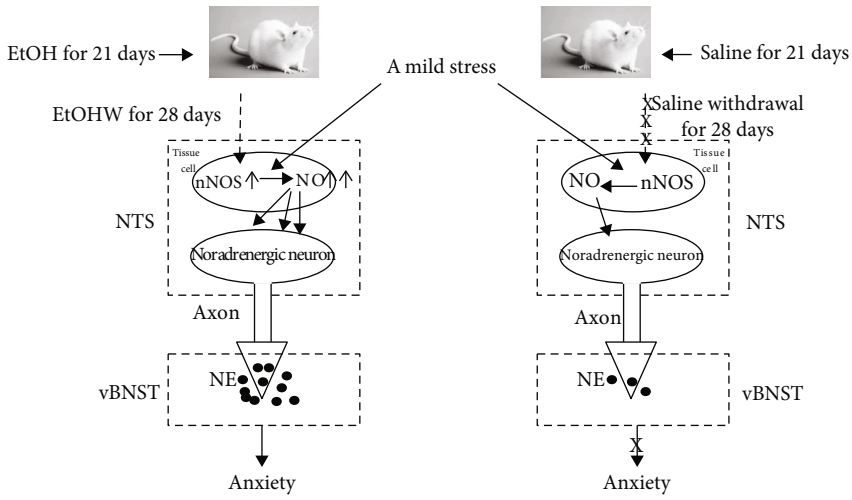

Figure 8: Mechanisms of AMRS-provoking anxiety during protracted EtOHW. EtOH: ethanol; EtOHW: EtOH withdrawal; NO: nitric oxide; nNOS: neuronal NO synthase; NE: norepinephrine; NTS: nucleus tractus solitarius; vBNST: ventral bed nucleus of the stria terminalis.

Both anxiety and plasma CORT secretion are closely associated with increased noradrenergic transmission in the BNST [12, 33]. In the present study, in vivo microdialysis showed that AMRS evoked sensitized NE release in the vBNST of EtOHW rats but not EtOH-naive rats, which is in line with the finding by Schmidt et al. [34] showing that mice experiencing repeated restraint stress exhibited elevated NE release in the BNST across multiple optogenetic stimulation parameters compared with stress-naive mice. However, pretreatment with carboxy-PTIO, L-NAME, or 7-NI, but not L-NIO, prevented the increase in the extracellular NE level. These results were consistent with the behavioral and hormonal findings in the present study and provide direct evidence of heightened NE release in the vBNST by stress during EtOHW. The increased NE release in the vBNST arises from noradrenergic excitation of both the soma region (NTS-A2) and the terminal area (vBNST) [24, 34]. Nevertheless, in the present study, the rate of

inhibition of NE release by intra-NTS carboxy-PTIO was ~80%, indicating that a large fraction of NE release was driven by excitation of NTS-A2 via NO signaling when challenged by AMRS during protracted EtOHW. These results suggest that inhibition of solitary NO signaling can attenuate AMRS-precipitated vBNST NE release, thereby mitigating anxiety during EtOHW.

The NTS primarily integrates and transmits visceral and external information to the forebrain, forming the autonomic-affective functional basis for the body. The gaseous molecule NO serves as both a neurotransmitter and neuromodulator, and is synthesized by three isoforms of NOS, i.e., nNOS, iNOS, and eNOS. We reported previously that systemic nicotine administration increased hypothalamic NE release via activation of both nNOS and eNOS in the NTS [25]. However, in the present study, L-NIO influenced neither anxiety nor NE release, which is consistent with the Western blot results that the EtOHW unaffected both eNOS and phospho-eNOS expressions in the NTS. By comparison, in the present study, both protein and mRNA levels of the solitary nNOS were increased during EtOHW; in addition, the solitary phospho-nNOS protein levels were significantly increased in EtOHW/AMRS rats but neither in EtOHW/non-AMRS rats nor in saline/AMRS rats. These results indicate that the nNOS system in the NTS during protracted EtOHW is in its "elevated response mode toward stress" and highly activated when challenged by AMRS, which sensitizes behavioral and noradrenergic response. This idea is also supported in the present study by the behavioral result that subsequent administration of SNP into the NTS abrogated the anxiolytic effect of 7-NI. Taken together, these findings reinforce the suggestion that solitary nNOS, but not eNOS, mediates the sensitized behavioral and neurochemical responses to AMRS during EtOHW. nNOS and eNOS are expressed in different types of cells in the NTS [35] that have different functions. For example, nNOS-derived NO induces glutamate release [36], whereas eNOS-derived NO enhances GABAergic transmission [37]. Thus, although the precise reasons have yet to be elucidated, these anatomical and physiological distinctions may at least partly account for the different effects of EtOHW on solitary nNOS and eNOS.

In summary, prior intra-NTS infusion of carboxy-PTIO, L-NAME, or 7-NI, but not L-NIO, attenuated the anxiety and vBNST NE release induced by 7-minute AMRS during EtOHW. EtOHW enhanced both nNOS protein and mRNA expression in the NTS but did not affect the eNOS protein level. These observations suggest that nNOS activity is promoted in the NTS during protracted EtOHW, which sensitizes the NTS-BNST noradrenergic response to stress and results in anxiety-like behavior in rats (Figure 8).

Data Availability

The data supporting the conclusions in this study are statistically analyzed and included in Results section and are available from the corresponding author on reasonable request.

Disclosure

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see http://www.textcheck.com/certificate/k6DTUj.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Rongjie Zhao, Zhenglin Zhao, and Sang Chan Kim designed the research. Zhenglin Zhao, Sang Chan Kim, Yu Jiao, Yefu Wang, and Bong Hyo Lee conducted the experiments. Chul Won Lee and Chae Ha Yang did the statistical analysis. Rongjie Zhao and Hee Young Kim wrote the manuscript. Zhenglin Zhao and Sang Chan Kim contributed equally to this work.

Acknowledgments

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References

[1] W. Dejong, "Relapse prevention: an emerging technology for promoting long-term drug abstinence," *International Journal of the Addictions*, vol. 29, no. 6, pp. 681–705, 1994.

[2] G. R. Breese, R. Sinha, and M. Heilig, "Chronic alcohol neuroadaptation and stress contribute to susceptibility for alcohol craving and relapse," *Pharmacology & Therapeutics*, vol. 129, no. 2, pp. 149–171, 2011.

[3] M. M. Weera and N. W. Gilpin, "Biobehavioral interactions between stress and alcohol," *Alcohol Research: Current Reviews*, vol. 40, no. 1, pp. e1–e7, 2019.

[4] G. F. Koob, "Alcoholism: allostasis and beyond," *Alcoholism: Clinical and Experimental Research*, vol. 27, no. 2, pp. 232–243, 2003.

[5] R. J. Smith and G. Aston-Jones, "Noradrenergic transmission in the extended amygdala: role in increased drug-seeking and relapse during protracted drug abstinence," *Brain Structure & Function*, vol. 213, no. 1-2, pp. 43–61, 2008.

[6] Z. L. Zhao, G. W. Zhao, H. Z. Li et al., "Acupuncture attenuates anxiety-like behavior by normalizing Amygdaloid Catecholamines during ethanol withdrawal in rats," *Evidence-based Complementary and Alternative Medicine*, vol. 2011, 8 pages, 2011.

[7] L. Albrechet-Souza, T. W. Viola, R. Grassi-Oliveira, K. A. Miczek, and R. M. M. de Almeida, "Corticotropin releasing factor in the bed nucleus of the Stria terminalis in socially defeated and non-stressed mice with a history of chronic alcohol intake," *Frontiers in Pharmacology*, vol. 8, 2017.

[8] D. Jadzic, V. Bassareo, A. R. Carta, and E. Carboni, "Nicotine, cocaine, amphetamine, morphine, and ethanol increase norepinephrine output in the bed nucleus of stria terminalis of

freely moving rats," *Addiction Biology*, vol. 26, no. 1, article e12864, 2021.

[9] J. A. Fuentealba, M. I. Forray, and K. Gysling, "Chronic morphine treatment and withdrawal increase extracellular levels of norepinephrine in the rat bed nucleus of the stria terminalis," *Journal of Neurochemistry*, vol. 75, no. 2, pp. 741–748, 2000.

[10] Z. A. McElligott, M. E. Fox, P. L. Walsh et al., "Noradrenergic synaptic function in the bed nucleus of the stria terminalis varies in animal models of anxiety and addiction," *Neuropsychopharmacology*, vol. 38, no. 9, pp. 1665–1673, 2013.

[11] G. R. Valdez, E. P. Zorrilla, A. J. Roberts, and G. F. Koob, "High-priority communication I Antagonism of corticotropin-releasing factor attenuates the enhanced responsiveness to stress observed during protracted ethanol abstinence," *Alcohol*, vol. 29, no. 2, pp. 55–60, 2003.

[12] M. I. Forray and K. Gysling, "Role of noradrenergic projections to the bed nucleus of the stria terminalis in the regulation of the hypothalamic-pituitary-adrenal axis," *Brain Research. Brain Research Reviews*, vol. 47, no. 1-3, pp. 145–160, 2004.

[13] C. F. Phelix, Z. Liposits, and W. K. Paull, "Monoamine innervation of bed nucleus of stria terminalis: an electron microscopic investigation," *Brain Research Bulletin*, vol. 28, no. 6, pp. 949–965, 1992.

[14] G. Aston-Jones, J. M. Delfs, J. Druhan, and Y. Zhu, "The bed nucleus of the stria terminalis. A target site for noradrenergic actions in opiate withdrawal," *Annals of the New York Academy of Sciences*, vol. 877, pp. 486–498, 1999.

[15] J. M. Delfs, Y. Zhu, J. P. Druhan, and G. Aston-Jones, "Noradrenaline in the ventral forebrain is critical for opiate withdrawal- induced aversion," *Nature*, vol. 403, no. 6768, pp. 430–434, 2000.

[16] R. L. Stornetta, F. E. Norton, and P. G. Guyenet, "Autonomic areas of rat brain exhibit increased Fos-like immunoreactivity during opiate withdrawal in rats," *Brain Research*, vol. 624, no. 1-2, pp. 19–28, 1993.

[17] L. H. Lin, "Glutamatergic neurons say NO in the nucleus tractus solitarii," *Journal of Chemical Neuroanatomy*, vol. 38, no. 3, pp. 154–165, 2009.

[18] V. Volke, S. Koks, E. Vasar, M. Bourin, J. Bradwejn, and P. T. Mannisto, "Inhibition of nitric oxide synthase causes anxiolytic-like behaviour in an elevated plus-maze," *Neuroreport*, vol. 6, no. 10, pp. 1413–1416, 1995.

[19] F. Yildiz, G. Ulak, B. F. Erden, and N. Gacar, "Anxiolytic-like effects of 7-nitroindazole in the rat plus-maze test," *Pharmacology Biochemistry and Behavior*, vol. 65, no. 2, pp. 199–202, 2000.

[20] Y. Jiao, S. C. Kim, Y. Wang et al., "Sauchinone blocks ethanol withdrawal-induced anxiety but spares locomotor sensitization: involvement of nitric oxide in the bed nucleus of the stria terminalis," *Evidence-based Complementary and Alternative Medicine*, vol. 2021, Article ID 6670212, 14 pages, 2021.

[21] V. T. Bonassoli, H. Milani, and R. M. de Oliveira, "Ethanol withdrawal activates nitric oxide-producing neurons in anxiety-related brain areas," *Alcohol*, vol. 45, no. 7, pp. 641–652, 2011.

[22] V. T. Bonassoli, E. B. Contardi, H. Milani, and R. M. de Oliveira, "Effects of nitric oxide synthase inhibition in the dorsolateral periaqueductal gray matter on ethanol withdrawal-induced anxiety-like behavior in rats," *Psychopharmacology*, vol. 228, no. 3, pp. 487–498, 2013.

[23] N. A. Gonzaga, M. R. Batistela, D. Padovan, B. S. de Martinis, C. R. Tirapelli, and C. M. Padovan, "Ethanol withdrawal induces anxiety-like effects: role of nitric oxide synthase in the dorsal raphe nucleus of rats," *Alcohol*, vol. 52, pp. 1–8, 2016.

[24] R. Zhao, H. Chen, and B. M. Sharp, "Nicotine-induced norepinephrine release in hypothalamic paraventricular nucleus and amygdala is mediated byN-Methyl-d-aspartate receptors and nitric oxide in the nucleus tractus solitarius," *Journal of Pharmacology and Experimental Therapeutics*, vol. 320, no. 2, pp. 837–844, 2007.

[25] H. F. Liu, Z. Zhao, J. Zhang et al., "Acupuncture reduces nicotine-induced norepinephrine release in the hypothalamus via the solitary NMDA receptor/NOS pathway," *Neuroscience Letters*, vol. 705, pp. 33–38, 2019.

[26] G. Paxinos and C. Watson, *The Rat Brain in Stereotaxic Coordinates*, Academic Press San Diego Calif USA, 1988.

[27] R. Sinha, H. C. Fox, K. I. Hong, J. Hansen, K. Tuit, and M. J. Kreek, "Effects of adrenal sensitivity, stress- and cue-induced craving, and anxiety on subsequent alcohol relapse and treatment outcomes," *Archives of General Psychiatry*, vol. 68, no. 9, pp. 942–952, 2011.

[28] A. Ostroumov, A. M. Thomas, B. A. Kimmey, J. S. Karsch, W. M. Doyon, and J. A. Dani, "Stress increases ethanol self-administration via a shift toward excitatory GABA signaling in the ventral tegmental area," *Neuron*, vol. 92, no. 2, pp. 493–504, 2016.

[29] C. Y. Shin, N. I. Lee, H. D. Je et al., "Cardiovascular responses and nitric oxide production in cerebral ischemic rats," *Archives of Pharmacal Research*, vol. 25, no. 5, pp. 697–703, 2002.

[30] M. Sanchez-Alavez, W. Nguyen, S. Mori et al., "Time course of microglia activation and brain and blood cytokine/chemokine levels following chronic ethanol exposure and protracted withdrawal in rats," *Alcohol*, vol. 76, pp. 37–45, 2019.

[31] B. A. Whitman, D. J. Knapp, D. F. Werner, F. T. Crews, and G. R. Breese, "The cytokine mRNA increase induced by Withdrawal from chronic ethanol in the sterile environment of brain is mediated by CRF and HMGB1 release," *Alcoholism: Clinical and Experimental Research*, vol. 37, no. 12, pp. 2086–2097, 2013.

[32] D. J. Knapp, B. A. Whitman, T. A. Wills et al., "Cytokine involvement in stress may depend on corticotrophin releasing factor to sensitize ethanol withdrawal anxiety," *Brain Behavior and Immunity*, vol. 25, pp. s146–s154, 2011.

[33] M. Cecchi, H. Khoshbouei, M. Javors, and D. A. Morilak, "Modulatory effects of norepinephrine in the lateral bed nucleus of the stria terminalis on behavioral and neuroendocrine responses to acute stress," *Neuroscience*, vol. 112, no. 1, pp. 13–21, 2002.

[34] K. T. Schmidt, V. H. Makhijani, K. M. Boyt et al., "Stress-induced alterations of norepinephrine release in the bed nucleus of the Stria terminalis of mice," *ACS Chemical Neuroscience*, vol. 10, no. 4, pp. 1908–1914, 2019.

[35] L. H. Lin, O. Taktakishvili, and W. T. Talman, "Identification and localization of cell types that express endothelial and neuronal nitric oxide synthase in the rat nucleus tractus solitarii," *Brain Research*, vol. 1171, pp. 42–51, 2007.

[36] W. T. Talman and D. N. Dragon, "Transmission of arterial baroreflex signals depends on neuronal nitric oxide synthase," *Hypertension*, vol. 43, no. 4, pp. 820–824, 2004.

[37] J. F. R. Paton, S. Kasparov, and D. J. Paterson, "Nitric oxide and autonomic control of heart rate: a question of specificity," *Trends in Neurosciences*, vol. 25, no. 12, pp. 626–631, 2002.