BIOMOLECULES & THERAPEUTICS

MeBib Suppressed Methamphetamine Self-Administration Response via Inhibition of BDNF/ERK/CREB Signal Pathway in the Hippocampus

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Abstract

Methamphetamine (MA) is one of the most commonly abused drugs in the world by illegal drug users. Addiction to MA is a serious public health problem and effective therapies do not exist to date. It has also been reported that behavior induced by psychostimulants such as MA is related to histone deacetylase (HDAC). MeBib is an HDAC6 inhibitor derived from a benzimidazole scaffold. Many benzimidazole-containing compounds exhibit a wide range of pharmacological activity. In this study, we investigated whether HDAC6 inhibitor MeBib modulates the behavioral response in MA self-administered rats. Our results demonstrated that the number of active lever presses in MA self-administered rats was reduced by pretreatment with MeBib. In the hippocampus of rats, we also found MA administration promotes GluN2B, an NMDA receptor subunit, expression, which results in sequential activation of ERK/CREB/BDNF pathway, however, MeBib abrogated it. Collectively, we suggest that MeBib prevents the MA seeking response induced by MA administration and therefore, represents a potent candidate as an MA addiction inhibitor.

Key Words: Methamphetamine, Self-administration, MeBib, HDAC6 inhibitor, Hippocampus

INTRODUCTION

Methamphetamine (MA) is an amphetamine-based psychostimulant, which is one of the most common drugs abused by illicit drug users. In humans, chronic drug abuse leads to flaws in executive functioning, information processing, and memory (Scott *et al.*, 2007). MA addicts show the selective patterns of brain deterioration leading to memory impairments (Ersche *et al.*, 2006). In addition, MA poisoning is characterized by chronic relapsing disease, and memory impairment increases the risk of relapse (Simon *et al.*, 2004). It induces degeneration of the hippocampus in both human and animal models. The hippocampus is located in the temporal lobe of the brain, and plays an important role in cognitive function, including short-term memory, motivation, and emotional response (Kuczenski *et al.*, 2007; Galinato *et al.*, 2015). In addition, the hippocampus is a limbic structure that is associated with learning and memory, is one of the brain region that is stimulated by psy-

chostimulants (Zhang *et al.*, 2014), which suggests that it is an important structure underlying the MA modulation of the reward circuit (Ricoy and Martinez, 2009).

Several studies have focused on the role of glutamate in relation to MA addiction (Nash and Yamamoto, 1992; Abekawa *et al.*, 1994; Qi *et al.*, 2012). Researchers have shown that MA interacts with the glutamatergic system, particularly the N-methyl-d-aspartate (NMDA) receptor system (Moriguchi *et al.*, 2002; Yeh *et al.*, 2002). NMDA receptors (NMDARs) are heteromeric ligand-gated cation channels comprising a glycine-binding GluN1 subunit and various glutamate-binding subunits of the GluN2 subfamily. It has been reported that NMDARs play a key role in long-term potentiation, a major form of synaptic plasticity, which is an underlying neurobiological mechanism for behavioral sensitization (Berberich *et al.*, 2007). For example, repeated treatment with high doses of MA has been reported to damage dopaminergic terminals and increase extracellular glutamate concentrations (Thomas

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et al., 2004; Halpin *et al*., 2014). In other studies, repeated MA exposure increased both the extracellular concentrations of dopamine and glutamate in the hippocampus (Rocher and Gardier, 2001). It is also known that brain-derived neurotrophic factor (BDNF) is increased through stimulation of glutamate-acting receptors (Jing *et al*., 2017). BDNF binds to its receptor, Tyrosine receptor kinase B (Trk-B), which in turn enhances the cAMP response element binding protein (CREB) in neurons (Stucky *et al*., 2016). Transcription factor CREB, a main target of nuclear calcium signaling, plays an important role in neuron survival. Interestingly, activation of synaptic NMDA receptors mediates phosphorylation of CREB at Ser133, an important transcriptional regulatory residue. (Go *et al*., 2016; Ashabi *et al*., 2017). To date, the effects of MA exposure on glutamate increase in specific regions of brain are well known. However, studies on regulatory mechanisms of glutamate release have not been understood yet.

Histone deacetylases (HDACs) are classified into Class I (HDAC1, HDAC2, HDAC3 and HDAC8), Class II (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10), Class III (sirtuins, SIRT 1-7) and Class IV (HDAC11) (Seto and Yoshida, 2014). HDAC has been extensively studied for synaptic plasticity and long-term memory as the core of drug addiction (Vecsey *et al*., 2007). Recent studies reported that the behavior induced by psychostimulants such as MA is associated with histone deacetylases (HDACs) (Kalda *et al*., 2007; Cadet, 2016; Torres *et al*., 2016). In previous papers, MA was reported to increase H4 acetylation (H4K5ac and H4K8ac) at short intervals in rat NAc and ancestors (Cadet *et al*., 2013).

Accordingly, HDACs inhibitors represent important agents for the development of pharmacological therapies combating the negative effects of psychostimulant exposure. MA-induced deacetylation of α-tubulin, an indicator of cellular structural loss in endothelial cells was also prevented by treatment with HDAC inhibitors (Fernandes *et al*., 2015). Valproic acid, an HDAC inhibitor, has also been shown to block the effects of MA on glutamate receptors by abrogating the effects of HDAC1 and HDAC2 on histone H4 acetylation (Jayanthi *et al*., 2014).

MeBib is a HDAC6 inhibitor derived from the benzimidazole scaffold. Benzimidazole is a heterocyclic aromatic compound, consisting of a phenyl ring fused with an imidazole ring. Numerous benzimidazole-containing compounds have attracted the attention of drug discovery efforts due to a wide spectrum of pharmacological activities (Lazer *et al*., 1987; Easmon *et al*., 2001; Chen *et al*., 2013). Furthermore, it has been reported that molecular probes containing benzimidazole scaffolds penetrate the brain-brain barrier (Eckroat *et al*., 2013).

In this study, we investigated whether the HDAC6 inhibitor MeBib reduced the self-administration of MA in rats. We also investigated the effect of MeBib on the molecular mechanism of neurotransmission by glutamate receptors, which are induced by MA.

MATERIAL AND METHODS

Animals

In this study, male Sprague-Dawley rats (200-250 g, 8 weeks of age, Hyochang Company, Daegu, Korea) were used. Rats were individually housed for free access to food and water. In an animal room with a constant light and dark cycle, the temperature and the humidity were kept constantly at 21-23°C and 55-65% each. The MA self-administration study was conducted during their dark cycle. Experimental procedures and animal care were in accordance with the Institutional Animal Care and Use Committee at the Keimyung University (Daegu, Korea; EXP-IRB number: KM_2018-007; the date of approval: 12 June 2018) The experiments were carried out in accordance with the Keimyung university's scientific research guidelines and regulations.

Preparation of drugs

Methamphetamine (MA) was purchased from the Ministry of Food and Drug Safety (Daejeon, Korea). MeBib was purified and received from Dr. Seo Young-ho, a professor of the college of Pharmacy, Keimyung University. MA was dissolved in sterile saline. The purity of MeBib is >95%. MeBib was dissolved in 10% DMSO. MeBib (10 mg/kg/mL) and its vehicle (10% DMSO in saline) were injected intraperitoneally in rats.

Apparatus

Rats were tested in operant conditioning chambers (Med Associates, St. Albans, VT, USA). Each chamber was equipped with two response levers (4.8×1.9 cm), a cue light (3 W, 28 V), and a house light (3 W, 28 V). A cue light was located above each response lever. The floor of the chamber was lined with wood chip bedding and covered with a metal grid. The chambers were placed within a sound-attenuating wooden enclosure, equipped with a ventilation fan. A syringe in the infusion pump (Razel Scientific Instruments, Stamford, CT, USA) was connected to a fluid swivel (Med Associates) with Tygon tubing. A Tygon tubing shield with a metal spring connected the swivel to the animal's catheter cannula assembly.

Food training

45 mg food pellets (Bio-Sub, French Town, NJ, USA) were used for lever press acquisition. The rats were fasted for about 1 day and then trained to press the lever to obtain 45 mg food pellets for 1 h to facilitate the acquisition of self-administration under food restriction. Rats which consumed all 100 food pellets during the 3 consecutive sessions had passed the lever press training and had a preparation period for intravenous catheterization surgery. Rats were fed an unlimited amount of food and water in the home cage for at least 2 days prior to intravenous catheterization surgery.

Intravenous catheterization

Rats which had undergone lever press training were anesthetized with a pentobarbital anesthesia (50 mg/kg, i.p.) and the chronic indwelling jugular catheter (Instech Solomon, Plymouth Meeting, PA, USA) was implanted into the right jugular vein. Catheters were secured to the jugular vein with Mersilene surgical mesh (Ethicon Inc., Somerville, NJ, USA) and exteriorized via a skin incision in the animal's back through a 22-gauge stainless-steel cannula (Plastics One, Roanoke, VA, USA), fixed to the head assembly with dental cement, and secured with a Prolene surgical mesh (Ethicon Inc.). Daily washing was performed with 0.2 mL saline containing heparin (20 U/mL) and gentamicin sulfate (0.33 mg/mL) to prevent clogging of the catheter due to blood flow.

MA self-administration

Rats undergoing intravenous catheterization surgery were

subjected to a lever press response to MA (0.1 mg/kg per infusion over 5 s in a 0.1 mL volume) at a fixed rate of 1 (FR1) after a 1 week recovery period. When the active lever was pressed, the cue indicator of the active lever was turned on for 5 s and the house light was turned off. After saline or MA was injected, the house light was turned off for 10 s timeout. Presses on the inactive lever were recorded but had no programmed consequence. Typically, this required 14 days following the initiation of MA self-administration. First, we tried to find out whether MeBib is a drug that helps suppress the stability of MA induction. Thus, rats were pretreated with MeBib (10 mg/kg/mL) or vehicle by intraperitoneal injection 30 min before starting the self-administration test from the start date. As a criterion for MA dependency, self-administration experiments were performed until a stable response pattern was established at less than 10% variance in the total number of responses for three consecutive sessions. Subsequently, the rats were sacrificed and samples were collected 1 h after completion of the MA self-administration.

Food reinforcement test

We performed a food reinforcement experiments based on the previous study (Jang *et al*., 2015). To investigate the effect of MeBib on food-reinforced responding, responses to each lever were measured in food-trained rats. Rats were limited to food to maintain 85% of their initial body weight and trained to press 45 mg food pellets (FR-1 schedule; 1 min timeout) for 2 h daily sessions. When rats obtained a lever pattern with a stable pattern of less than 10% variance in the total number of pellets obtained for three consecutive days, MeBib (10 mg/kg) or saline was administered intraperitoneally 30 min prior to the food reinforcement test. The total number of active and inactive lever responses by food pellet delivery was measured during the 2 h session.

Western blot analysis

The brain was cut using a cryostat microtome and both the dorsal and ventral hippocampal tissues were taken and used for data analysis. These tissues were homogenized in protein extraction buffer containing protease and phosphatase inhibitors. Protein concentrations were measured using a Pierce™ BCA Protein assay kit (Thermo Scientific, Waltham, MA, USA). Equal amounts of total protein (30 μg) were separated on an 8% or 12% SDS-PAGE gel and transferred to a PVDF membrane (Merck Millipore, MA, USA). After being blocked in 5% milk for 1 h at room temperature, membranes were incubated overnight at 4°C with the following primary antibodies against AMPA receptor 2 (GluA2; 1:1000, 13607, Cell Signaling, Beverly, MA, USA), BDNF (1:1000, ab108319, Abcam, Cambridge, UK), phospho-CREB (ser133,1:1000, 9198, Cell Signaling, Beverly, MA, USA), CREB (1:1000, 4820, Cell Signaling) phospho-p44/42 MAPK (ERK1/2; 1:1000, 9101, Cell Signaling), p44/42 MAPK (ERK1/2; 1:1000, 9102, Cell Signaling), NMDA receptor2B (GluN2B; 1:1000, 4212, Cell Signaling), and β-Actin (1:1,000, 4970, Cell Signaling). Membranes were washed in TBST for 20 min and probed with Horseradish Peroxidase (HRP) conjugated secondary antibodies: Anti-Mouse IgG (1:5,000, 7076, Cell Signaling) and Goat Anti-Rabbit IgG (1:5,000, 7076, Cell Signaling); Rabbit Anti-Goat IgG H&L (HRP) (ab6741, Abcam). After chemiluminescent substrate (Amersham Bioscience, Buckinghamshire, UK) was distributed on the membrane, images were captured

using a Fusion SOLO S system (Vilber Lourmat, Eberhardzell, Germany). Protein bands were quantified with ImageJ software (National Institute of Mental Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij). The values were normalized to β-actin intensity levels.

Statistics

Statistical significances between groups were determined using the two-way analysis of variance (ANOVA) with repeated measures (RM), followed by Bonferroni's post hoc test, respectively. Analysis was conducted using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Data were presented as mean ± SEM for each group (n=6 per group). Statistical significance was accepted for *p* values<0.05.

RESULTS

MeBib inhibits continuous MA self-administration

Rats were divided into 4 groups (n=6). On each of 14 consecutive days, rats were allowed to acquire a lever press response for MA (0.1 mg/kg per infusion over 5 s in a 0.1 mL volume) under a fixed-ratio 1 (FR1) schedule. When the active lever is pressed, the cue light above the active lever turns on for 5 s, the house light turns off. After each infusion, an additional timeout period of 10 s was added with the house light off. Pressing of the inactive lever is recorded, but no programmed result. The daily averages for active lever presses, inactive lever presses, and infusions for MA self-administering rats over a 14-day period are shown in Fig. 1. The MA self-administered rat group (MA group) showed a higher MA seeking behavior with a significant number of infusion lever presses compared to the control group ($F_{13,65}$=5.781, *p*<0.0001), MeBib group ($F_{13,65}$=9.330, *p*<0.0001), MeBib and MA group ($F_{13,65}$=9.330, *p*<0.001) (Fig. 1B). The frequencies of inactive lever presses were similar in all groups (Fig. 1C). MA maintained robust self-administration behavior, whereas the group exposed to saline or MeBib did not. All animals self-administering MA showed significantly higher rates of response with the active lever compared with controls ($F_{13,65}$=5.886, *p*<0.0001). Daily intraperitoneal administration of MeBib for 30 min before the beginning of self-administration session resulted in a significant reduction in MA intake ($F_{13,65}$=2.373, *p*<0.001) (Fig. 1D). Thus, MeBib appeared to regulate the MA seeking response.

MeBib did not affect food reinforcement training

To test whether MeBib affects generalized behavioral responses, food reinforcement training was conducted using sucrose pellets. MeBib (10 mg/kg) and its vehicle (10% DMSO in saline) were injected intraperitoneally 30 min before the food reinforcement test. As shown in Fig. 2, MeBib did not affect food reinforcement training ($F_{1,28}$=0.08738, *p*=0.7697).

MeBib inhibits the expression of GluN2B, an NMDA receptor subunit

Glutamate release by MA is known to activate N-methyl-d-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors that induce excitotoxicity (Simoes *et al*., 2008). Western blot analysis was used to determine the protein expression of the NMDA receptor subunit GluN2B (Fig. 3B) and the AMPA receptor subunit GluA2 (Fig. 3A) in each group. MA administration significantly
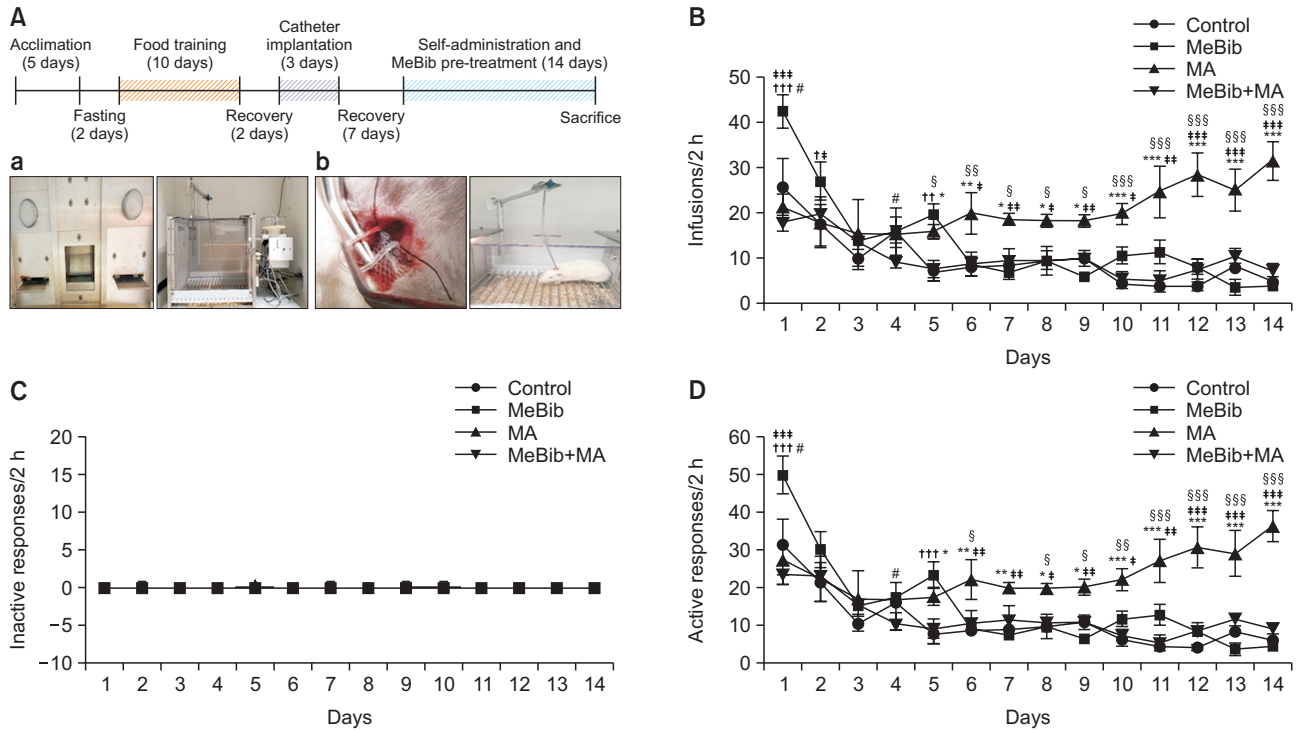
Fig. 1. MeBib inhibits continuous MA self-administration. (A) Experimental procedure. a. Lever and food pellet supply system, b. Mouse implanted with a catheter. (B) The mean number of infusion lever presses (± SEM) during the 14-day experiment. (C) The graph shows mean number of inactive lever presses (± SEM). (D) The graph shows mean number of active lever presses (± SEM). The active lever press includes FR1 10 s timeout and the number of 5 s that methamphetamine is injected. Data were presented as mean ± SEM for each group (n=6 per group). *$p<0.05$, **$p<0.01$, ***$p<0.001$ control vs MA; †$p<0.05$, ††$p<0.01$, †††$p<0.001$ control vs MeBib; #$p<0.05$ control vs MeBib+MA; ‡$p<0.05$, ‡‡$p<0.01$, ‡‡‡$p<0.001$ MeBib vs MA; §$p<0.05$, §§$p<0.01$, §§§$p<0.001$ MA vs MeBib+MA. First, the degrees of freedom and the graph associated with Fig. 2 do not match - specifically, the degrees of freedom look like they would be associated with an acquisition curve, while the data provided are collapsed into single points, which would greatly reduce the degrees of freedom. The statistics should reflect the data that are shown.
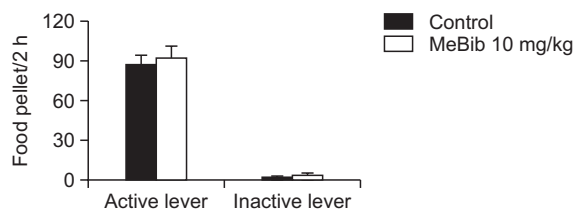

Fig. 2. MeBib did not affect food reinforcement training. The figure shows the total number of responses for the active lever (left) and inactive lever (right) for 2 h. MeBib (10 mg/kg) or saline was injected intraperitoneally 30 min before starting the food self-measurement. There was no significant difference between the saline and MeBib groups in the total number of reactions for the active and inactive levers for 2 h. The 'food pellets' on the y axis represent the average number of lever presses of food pellets over 2 h. n=8 per group.

increased the levels of GluA2 ($F_{6,24}=7.730$, $p=0.0001$) and GluN2B ($F_{6,24}=9.233$, $p<0.0001$) expression compared with control. However, the group treated with MeBib showed a decrease in GluN2B levels compared with the group treated with MA alone. These results suggest that the MeBib inhibited the self-administration of MA via regulation of GluN2B.

MeBib reduces ERK activation by MA self-administration

According to previous studies, the hippocampus participates in the formation and consolidation of new memories mediated via MAPK and ERK (Cammarota *et al.*, 2008). It is also known that the ERK pathway is activated by GluN2B-containing receptors (Krapivinsky *et al.*, 2003). Thus, we investigated whether MeBib regulated the phosphorylation of ERK via GluN2B inhibition in the MA-administered group. Our results showed that ERK phosphorylation was upregulated by MA self-administration ($F_{6,24}=5.004$, $p=0.0019$), and this activation was inhibited by pretreatment of MeBib (Fig. 4A).

MeBib inhibits CREB phosphorylation by MA self-administration

CREB signaling pathway, including the NMDA receptor, plays an important role in MA-induced behavioral sensitization and rewarding effects (Nestler, 2001; Brami-Cherrier *et al.*, 2005). Therefore, we elucidated the effect of MA self-administration on CREB phosphorylation. We found that CREB phosphorylation was increased in the MA-treated group ($F_{6,24}=7.355$, $p=0.0002$); however, it was also inhibited by MeBib treatment (Fig. 4B).

MeBib inhibits BDNF expression by MA self-administration

Drug-induced changes in serum brain-derived neurotrophic factor (BDNF) in the cortex and hippocampus have been ob-
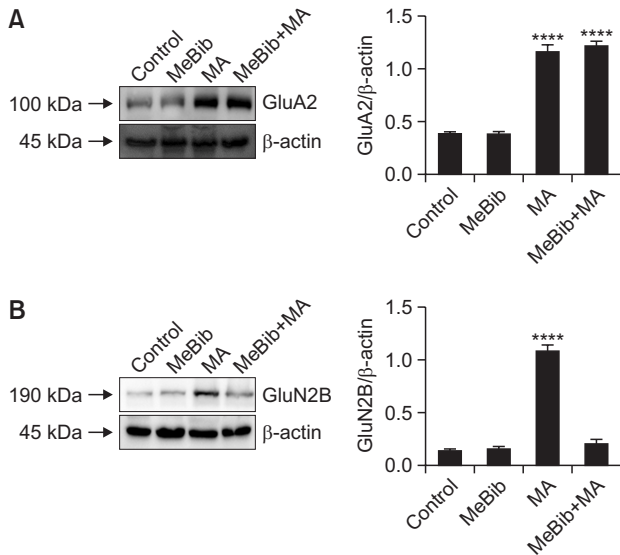
A

100 kDa → GluA2
45 kDa → β-actin

B

190 kDa → GluN2B
45 kDa → β-actin

Fig. 3. MeBib inhibits the expression of GluN2B, an NMDA receptor subunit. (A, B) The hippocampal tissues from each group was subjected to western blot analysis. Membranes were incubated with GluA2 or GluN2B antibodies. β-actin was used to confirm equal sample loading. Data are representative of three independent experiments and quantified as mean ± SEM. Two-way ANOVA, followed by Bonferroni's post hoc test. ****$p<0.0001$ compared to control.

A

44 kDa → 42 kDa → p-ERK
44 kDa → 42 kDa → ERK
45 kDa → β-actin

B

43 kDa → p-CREB
43 kDa → CREB
45 kDa → β-actin

C

15 kDa → BDNF
45 kDa → β-actin

Fig. 4. MeBib inhibits ERK, CREB and BDNF expression by MA self-administration. (A-C) The hippocampal tissues from each group was subjected to western blot analysis. Membranes were incubated with p-ERK, ERK, p-CREB and BDNF antibodies. β-actin was used to confirm equal sample loading. Data are representative of three independent experiments and quantified as mean ± SEM. Two-way ANOVA, followed by Bonferroni's post hoc test. ****$p<0.0001$ compared to control.

served in MA users (Kim *et al*., 2005). Additionally, increased levels of BDNF were associated with enhanced NMDA expression and intracellular calcium concentrations (Walz *et al*., 2017). Thus, we investigated whether modulation of GluN2B expression by MeBib affected BDNF expression. As shown in Fig. 4C, the increase in BDNF expression by MA self-administration ($F_{6,24}=4.893$, $p=0.0021$) was inhibited by MeBib treatment.

DISCUSSION

MA is one of the most commonly abused drugs by illicit drug users worldwide. Many studies reported that MA abuse leads to cognitive impairment, and memory deficits. Besides, MA induces neuronal degeneration of the brain area linked to cognitive function. Therefore, understanding the synaptic changes and neuronal mechanisms that are affected by MA exposure has become imperative. In this study, we elucidated the effects of HDAC6 inhibitor MeBib on MA self-administration. Our results demonstrated that the number of active lever presses in MA self-administered rats was reduced by pretreatment with MeBib, which also suppressed the expression of the NMDA receptor GluN2B and inhibited the ERK/CREB/BDNF signaling pathway.

We found that the expression of GluA2 and GluN2B was increased in the hippocampus of MA self-administered rats. These data are consistent with previous reports suggesting that the expression of NMDA receptor was regulated after MA exposure and modulated the excitotoxic effect (Bowyer and Ali, 2006; Kalivas and Volkow, 2011). Another study also reported that MA self-administration increased the NMDA receptor currents and surface expression of the GluN2B subunit (Mishra
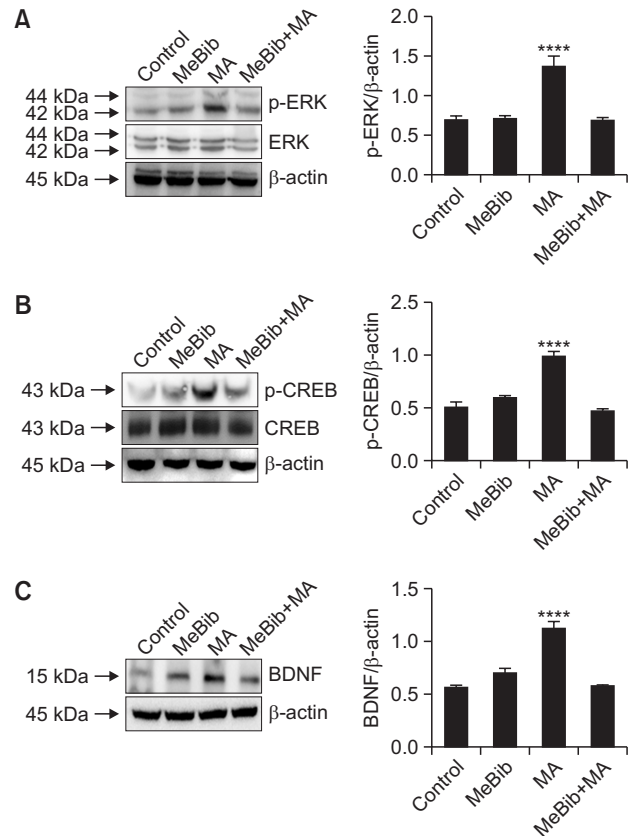
et al., 2017). In addition, the expression of GluN2B by MA is considered one of the fundamental mechanisms affected by addictive drugs (Galinato *et al*., 2015; Mishra *et al*., 2017). These studies suggest that the overexpression of GluN2B is an important factor in MA intoxication. Furthermore, several studies have shown that HDAC inhibitors regulate glutamate receptor expression. (Bhattacharya *et al*., 2017; Xing *et al*., 2019), however, the molecular mechanisms are poorly understood. We found for the first time MeBib, a HDAC6 inhibitor, suppressed the MA seeking response. And, we also observed that GluN2B expression was inhibited when the MA self-administered group was pretreated with MeBib. Therefore, the inhibition of GluN2B expression by MeBib treatment suggests that MeBib represents an important agent for the modulation of MA addiction. However, molecular mechanisms underlying HDAC6 inhibitor inhibits drug seeking response should be further elucidated.

Previously, researchers reported that ERK activation occurs as a calcium-dependent NMDAR-mediated response, which transmits extracellular stimuli to the nucleus and regulates synaptic plasticity and learning (Huo *et al*., 2014). Also, behavioral and molecular responses induced by MA regulate
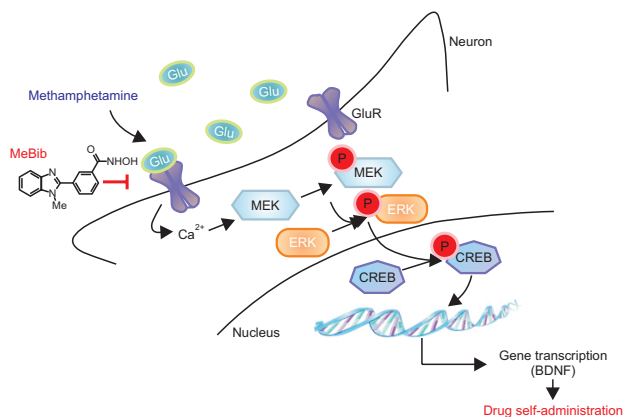
Fig. 5. Schematic diagram for potential pharmacological mechanisms of MeBib.

the activity of the ERK pathway and depend on NMDA receptor interactions to modulate gene expression, plasticity, and behavior (Pickens *et al*., 2011; Galinato *et al*., 2015). Furthermore, GluN2B-containing NMDAR and its downstream ERK pathway have been implicated in MA-induced behavioral sensitization (Li *et al*., 2016). Consistent with these studies, our results showed that ERK phosphorylation was increased in the MA group, which was inhibited by MeBib pretreatment.

Next, we found that CREB, one of the most important transcription factors mediating various neuronal connections (Sakamoto *et al*., 2011), was involved in MA addiction. Several reports suggest that ERK regulated CREB phosphorylation (Mizoguchi *et al*., 2004), and MA modulated the ERK/CREB pathway, which is related to changes in spatial memory (Cao *et al*., 2013). As shown in Fig. 3B, the upregulation of CREB phosphorylation in the MA group was inhibited by MeBib.

BDNF release in the hippocampus increases tyrosine phosphorylation of the GluN2B subunit at Tyr-1472, which is a critical step in the stimulation of synaptic transmission (Lin *et al*., 1998; Caldeira *et al*., 2007). Moreover, BDNF regulates drug addiction-related behaviors by modulating the dopamine system and other compensating regions (Galinato *et al*., 2015), and the increase in GluN2B tyrosine phosphorylation by BDNF was comparable to the levels observed after hippocampal long-term potentiation (LTP) induction (Rosenblum *et al*., 1996) (Rostas *et al*., 1996). It has been reported that MA-treated animals showed augmented BDNF levels in the hippocampus (Skelton *et al*., 2007; Grace *et al*., 2008). Similarly, our western blot analysis showed that BDNF expression was increased in the MA self-administration group, which was inhibited by pretreatment with MeBib. Therefore, we speculate that the increased GluN2B level following MA exposure was mediated by the overexpression of BDNF, and MeBib abrogated this signal transduction pathway.

Collectively, we found that MA self-administration induced GluN2B expression, associated with the activation of sequential ERK/CREB/BDNF pathway in an animal model; however, it was suppressed by HDAC6 inhibitor MeBib (Fig. 5). Thus, we suggest that MeBib prevents MA seeking response induced by MA and therefore, represents a potent candidate as an MA addiction inhibitor.

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