Regulatory Effect of Bee Venom on Methamphetamine-Induced Cellular Activities in Prefrontal Cortex and Nucleus Accumbens in Mice

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Our previous studies demonstrated that subcutaneous injection of bee venom (BV) into the Zusanli (ST36) acupuncture point, namely BV acupuncture, dose-dependently prevents conditioned place preference (CPP) induced by repeated injection of methamphetamine (METH) in mice. To expand on our observations, the present study was designed to determine the suppressive mechanisms of BV acupuncture in the development of METH-induced CPP by evaluating the changes in expression of ΔFosB, phosphorylated extracellular signal-regulated kinase 1/2 (pERK), and phosphorylated calcium/calmodulin-dependent protein kinase type II (pCaMKII) in the prefrontal cortex (PFC) and nucleus accumbens (NAc) in mice. Pre-emptive treatment with BV at 30 min before repeated METH injection completely suppressed acquisition of CPP at the day 7 test session. METH-induced upregulation of ΔFosB and pERK in PFC and NAc was significantly reduced by BV pretreatment. Expression of pCaMKII was significantly elevated by METH in NAc and reduced in PFC. BV pretreatment reversed the changes of pCaMKII expression in PFC and NAc. These findings suggest that BV acupuncture may exert a suppressive effect on METH-induced addiction via regulation of signaling cascades of ΔFosB, ERK, and CaMKII in PFC and NAc.

Key words methamphetamine (METH); bee venom (BV); conditioned place preference (CPP); prefrontal cortex (PFC); nucleus accumbens (NAc)

Addiction to methamphetamine (METH) is an international public health problem. Acute administration of METH results in a sense of euphoria and hyperactivity in association with increased extracellular release of monoamine neurotransmitters, including dopamine, norepinephrine and serotonin. Chronic METH abuse produces several negative side effects, including dopaminergic nerve terminal toxicity, altered morphology in the central nervous systems and an increased risk of developing Parkinson’s disease. Nevertheless, exacerbating the problem of METH-induced neurotoxicity is the current lack of Food and Drug Administration (FDA)-approved pharmacotherapies for treating the negative health effects of METH abuse.

Numerous studies have been performed to evaluate the most effective therapeutic methods to treat METH addiction through the long-lasting prevention of relapse to METH use without side effect. Some of these studies have investigated needle acupuncture, which has been used to treat various drug addictions in humans. However, most studies revealed that needle acupuncture produces a significant but only partial therapeutic effect compared with the placebo control. Our recent studies firstly demonstrated that acupuncture with bee venom (BV), which is a potent peripheral stimulant that is dissolved in saline and administered into Zusanli acupuncture point (ST36), could dose-dependently reduce the hyperactivity induced by a single injection of METH as compared with other acupuncture points [Yinlingquán (SP9) and Xuanzhong (GB39)] or non-acupuncture point (tail base). This result indicated that BV injection into Zusanli acupuncture point was a potent method for the suppression of METH addiction. On the other hand, repeated METH (1 mg/kg) administration at days 1, 3 and 5 produced development of conditioned place preference (CPP) on day 7 (acquisition state). Following acquisition of METH for 6 d, CPP was completely gone at day 17 (extinction state). On day 18, the sub-effective dose of METH (0.1 mg/kg) re-increased CPP with similar level of acquisition stage (reinstatement state). We observed that pre-treatment of BV acupuncture before METH injection during acquisition stage completely inhibited development of CPP in both acquisition state and reinstatement state, whereas single pre-treatment of BV acupuncture after acquisition state did not affect the development of CPP at reinstatement stage. Although precise mechanisms of BV acupuncture in the development of CPP at acquisition state are still unclear, these results strongly indicate that pre-emptive treatment of BV acupuncture may be a promising way to support treatments for METH dependence. In this reason, present study was aimed to address inhibitory mechanism underlying pre-emptive BV acupuncture in the development of CPP by METH. METH addicts may be related to neurological changes in the prefrontal cortex (PFC) and its glutamatergic projections to the nucleus accumbens (NAc). A recent study shows some initial characterization of the modifications of METH-induced intracellular signaling cascades and associated gene transcription factors in these regions of the brain. The development and expression of METH-induced locomotor sensitization and psychological changes accompanied increased levels of phosphorylated extracellular signal-regulated kinase 1/2 (pERK) in the brain areas. In contrast, the level of phosphorylated calcium/calmodulin-dependent protein kinase type II (pCaMKII), which has been implicated in the regulation of dopaminergic neurotransmission, decreased in brain regions after chronic treatment.
administration of METH. Moreover, it is notable that the increased level of ΔFosB, which is a long-lasting transcription factor, is involved in METH-induced neurotoxicity in the central nervous system. Our previous study revealed that a single METH injection significantly increased neuronal activation (Fos expression) in these brain regions, and this activation was regulated by BV acupuncture. As an extension of our findings, the current study was designed to determine the intracellular suppressive mechanisms of pre-emptive BV acupuncture in discrete brain areas, including the PFC and NAc, following repeated METH injection.

MATERIALS AND METHODS

Animals Experiments were performed using male ICR mice (20–22 g). All experimental animals were purchased from the Daehan Biolink (Chungbuk, South Korea) and housed in colony cages with free access to food and water and maintained in a temperature- and light-controlled room (23°C, 12/12 h light/dark cycle with lights on at 08:00) for at least 1 week prior to the study. All of the methods used in the present study were approved by the Institute of Animal Care and Use Committee at Chonbuk National University and conform to NIH guidelines (NIH Publication No. 86-23, revised in 1985).

Procedure for Conditioned Place Preference For place conditioning, eight identical polyvinyl chloride shuttle boxes (30 cm × 20 cm × 30 cm), each divided by a separator into two chambers of equal size (30 cm × 20 cm × 15 cm), were used. One chamber was black with a smooth floor and the other was white with a wire mesh floor. Each test animal received a pre-exposure test for 3 d before the experiment, in which they were allowed access to the entire apparatus for 15 min. The amount of time spent (s) in each chamber was monitored. On days 1, 3 and 5 mice were subcutaneously given METH (1 mg/kg) and then placed in the assigned chamber for 60 min. On days 2, 4 and 6, mice were given saline before being confined in the other chamber for 60 min. On the test day (day 7), the door separating the two chambers was opened again, and the mice were allowed free access to the entire apparatus for 15 min without any restriction. An acquisition test, in which the amount of time spent (s) in each chamber was recorded, was performed (Fig. 1A). The difference in seconds between the time spent in the drug-paired compartment in the post-conditioning test (day 7) and that spent in the same compartment in the pre-conditioning day (day 0) is a measurement of conditioned place preference (CPP, s) caused by the drug. The behavior of the mice was recorded by a video camera and analyzed by Smart-CS system set (Panlab, Barcelona, Spain).

Bee Venom Treatment BV from Apis mellifera (Cat. No.: V3375, Sigma, St. Louis, MO, U.S.A.) was dissolved in a sterile saline solution at 1 mg/mL, which dose of BV produced most potent preventive effect on METH induced CPP as previously described. Twenty microliter of BV solution was bilaterally and subcutaneously administrated into the Zusanli acupuncture point (ST36) at 30 min prior to METH injection on days 1, 3 and 5, as previously described. As a BV acupuncture control group, a vehicle control group received an injection of a corresponding volume of saline in same site.

Western Blotting After the CPP test (on day 7), the mice were killed by decapitation and each brain region (prefrontal cortex; PFC, nucleus accumbens; NAc) was removed in less than 60 s and was quick-frozen on dry ice, weighed, and then stored at −80°C. Each brain sample was homogenized in buffer containing 1% Tris (pH 7.5), 1% NP-40, 0.5 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.5), 50 mM ethylene glycol bis[2-aminoethyl ether]-N,N′,N′,N′-tetraacetic acid (EGTA), 1 mM dithiothreitol, 1 mM benzamidine and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The total amount of protein in each sample was determined using the Bradford assay before loading the proteins into polyacrylamide gels. The homogenates (50 μg protein) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were then transferred to nitrocellulose membranes. After the blots had been washed with TBST (10 mM Tris–HCl [pH 7.6], 150 mM NaCl, and 0.05% Tween-20), the membranes were blocked with 5% skim milk for 1 h and then they were incubated with the appropriate primary antibodies for ΔFosB (1:500; Abcam, MA, U.S.A.), calcium/calmodulin-dependent protein kinase type II (CaMKII, 1:1000; Abcam), phosphorylated calcium/calmodulin-dependent protein kinase type II (pCaMKII, 1:500; Santa Cruz, CA, U.S.A.), extracellular signal-regulated kinase 1/2 (ERK, 1:1000, Cell Signaling Technologies, MA, U.S.A.), phosphorylated extracellular signal-regulated kinase 1/2 (pERK, 1:1000, Cell Signaling Technologies) and β-actin/α-tubulin (loading control; Sigma). The membranes were then washed, the primary antibodies were detected using the appropriate secondary immunoglobulin G conjugated to horseradish peroxidase, and the bands were subsequently visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, Uppsala, Sweden).

Data and Statistical Analysis Data values were ex-
expressed as the mean±S.E.M. All data were analyzed using the commercially available software GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, U.S.A.). Statistical analysis was carried out using one-way ANOVA followed by Tukey’s multiple comparisons test.

RESULTS

The time spent in the drug-paired chamber (CPP score) was significantly higher in the group treated with repeated METH administration at days 1, 3 and 5 (METH–Sal group) as compared with the saline-injected group (Sal–Sal group) on day 7 (Fig. 1B). Similar to our previous finding, 7 pre-emptive treatment with BV (20 µL with BV 1 mg/mL concentration) prior to METH injection (METH–BV group) completely suppressed the acquisition of CPP at the day 7 test session, as compared with the METH–Sal group [Fig. 1B, F(2, 15)=43.33, p<0.0001]. To be able to determine whether the BV altered the METH-induced neuronal activity of the PFC and NAc, brain tissues were obtained after CPP test. As compared with Sal–Sal group, METH–Sal group show significant elevation of ΔFosB in several brain areas as similar with our previous finding. 6 Generally, ΔFosB is enhanced in the NAc and its target site (ventral pallidum) after repeated METH injection. 18 Present studies have demonstrated that METH significantly increases neuronal activity by increasing Fos and ΔFos expression in several brain areas as similar with our previous finding. 5 Generally, ΔFosB is enhanced in the NAc and its target site (ventral pallidum) after repeated METH injection. 18 In this study, repeated administration of METH caused profound enhancement of ΔFosB expression in the PFC and NAc, accompanying CPP. Pre-emptive treatment with BV acupuncature significantly attenuated the expression of ΔFosB in these brain areas and evoked CPP due to repeated METH exposure, suggesting that BV acupuncture has a potential suppressive effect on the development of METH-induced CPP, and that the underlying molecular mechanism of BV might be related to its regulatory effect in the PFC and NAc, as previously described. 17

It is well known that glutamatergic dysfunction may impact dopaminergic transmission, and ultimately lead to the emergence of psychosis in METH abuse. 19 ERK, a member of the MAPK family, is linked to dopaminergic neurotransmission

![Fig. 2. Western Blotting Image (A) and Analysis Data (B) for the Changes in ΔFosB Expression in the Prefrontal Cortex (PFC) and Nucleus Accumbens (NAc) after the Injection of Saline (Sal), Methamphetamine (METH), and METH+Be Venom (BV) Acupuncture at Day 7](image1)

![Fig. 3. Western Blotting Image (A) and Analysis Data (B) for the Changes in Extracellular Signal-Regulated Kinase 1/2 (ERK) and Phosphorylated ERK (pERK) Expression in the Prefrontal Cortex (PFC) and Nucleus Accumbens (NAc) after the Injection of Saline (Sal), Methamphetamine (METH), and METH+Be Venom (BV) Acupuncture at Day 7](image2)
The present study also revealed that BV acupuncture dramatically reversed the METH-induced CPP and the activation of the dopamine D1 receptor. Although the mechanisms underlying BV acupuncture-induced improvements relating to METH addiction may be related to the development of CPP, induced by METH, may be related to the activation of ERK and CaMKII via dopamine D1 receptors. In the present study, we observed that METH-induced CPP was accompanied by the activation of ERK and CaMKII phosphorylation in the NAc. Moreover, pre-emptive treatment of BV acupuncture completely inhibited METH-induced ERK and CaMKII activation. Therefore, it is assumed that the suppressive effect of BV acupuncture may be produced by its inhibitory effect on ERK and CaMKII signaling cascades in the NAc. In conclusion, the present study demonstrated for the first time that the signaling pathway cascade including ΔFosB, ERK and CaMKII in the PFC and the NAc is crucial, at least in part, to the long-lasting neuro-adaptation induced by METH, which may be related to the suppressive effects of pre-emptive BV acupuncture. Although the mechanisms underlying BV acupuncture-induced improvements relating to the extracellular release of monoamine neurotransmitters in the brain have yet to be delineated, our results support the notion that BV acupuncture represents an effective intervention aimed at repairing brain dopaminergic abnormality caused by repeated METH exposure.

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**Conflict of Interest** The authors declare no conflict of interest.

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