Acute administration of methamphetamine decreases the mRNA expression of diazepam binding inhibitor in rat brain

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Chronic administration of methamphetamine (MAP) up-regulated the mRNA expression of diazepam binding inhibitor (DBI) in rat brain, possibly leading to anxiety. Acute effects of MAP on anxiety associated with DBI, however, are not clear. In this study, we examined the effects of acute administration of MAP on behavior related to anxiety and the expression level of DBI mRNA and pituitary adenylate cyclase-activating polypeptide (PACAP) mRNA, calibrated with the glyceraldehydes 3-phosphate dehydrogenase mRNA as the internal control in rat brain. The elevated plus-maze test was applied to the analysis of the possible anxiety-related profile of MAP. Acute administration of MAP (5 mg/kg, intraperitoneal administration) significantly increased spent time in the open-space arms at 4 h after the administration compared with a saline-treated group. The expression of DBI mRNA in a large number of regions of rat brain significantly decreased 2, 4, 8 and 16 h after acute administration of MAP. In contrast, the expression of PACAP mRNA in a large number of regions of rat brain significantly increased 4 and 8 h after the administration of MAP. These results suggest that MAP, at this dose, has an anxiolytic effect, based on the reduction of the putative anxiogenic peptides, DBI.

Key words: methamphetamine, anxiety, elevated plus-maze test, diazepam binding inhibitor, pituitary adenylate cyclase-activating polypeptide

INTRODUCTION

Anxiety is one of the common symptoms in the patients suffering from dependence and withdrawal syndrome by abuse-inducing drugs such as morphine, ethanol and methamphetamine. Diazepam binding inhibitor (DBI), an approximately 10 kDa polypeptides distributed in both the central and peripheral nervous system [1, 2, 8, 23], is an anxiogenic factor during drug dependence. With some behavioral and pharmacological studies, DBI was found to be an inverse agonist for the type A receptor for the gamma-aminobutyric acid ($GABA_A$ receptor) [10] as well as an endogenous agonist for the peripheral-type benzodiazepine receptors (PBR) to stimulate steroid synthesis in the mitochondria [18]. Injection of DBI into the lateral ventricle induced anxiety [5, 10, 23] and an elevation in the expression level of DBI peptides in brain induced behavioral changes, including anxiety [7, 10, 19]. On the basis of these results, DBI mRNA increases in the cerebral cortex of mouse showing dependence on morphine [12], ethanol [11] and nicotine [13] in relation to anxiety. In addition, our previous study demonstrated that the expression level of DBI mRNA in the several regions of rat brain after chronic administration of methamphetamine (MAP) increases, corresponding with changes of expression of the DBI-related mRNA such as PBR, the $GABA_A$ receptor alpha2 subunit and the pituitary adenylate cyclase-activating polypeptide (PACAP), which are assumed to collaborate with each other to make a pathway, finally leading to anxiety [27]. These results support the anxiogenic property of DBI.

Numerous studies shown that acute exposure of MAP has a psychostimulant (including anti-anxiety) and sympathomimetic effects, while chronic exposure of MAP causes the dependence and withdrawal symptoms (including anxiety) [3, 6, 16, 20]. In addition, the exposure of cultured astrocytes to pituitary adenylate cyclase-activating polypeptide (PACAP) for 6 h in a dose dependent manner stimulates the expression of DBI mRNA and releases DBI through activation for the receptor of pituitary adenylate cyclase-activating polypeptide (PAC1-R) [15]. To elucidate the effects of acute administration of MAP associated with anxiety in central nervous system, we studied relationship between the behavior and the expression of mRNA of DBI and PACAP in rat brain in relation to anxiety using the elevated plus-maze test which is routinely used to study anxiety-related behavior in rats and mice [17, 21], and the real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method for

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the analysis of the expression level of DBI and PACAP mRNA in the several regions of rat brain after acute administration of MAP.

MATERIALS AND METHODS

Animals and materials

The present animal experiments were performed in strict accordance with the guidelines for Animal Experimentation of Tokai University, and were approved by the Animal Experimentation Committee of Tokai University. Male Wistar rats (Clea Japan, Tokyo, Japan) weighing 200 to 250 g were group-housed in laboratory cages and kept in a temperature-controlled room (23 ± 2°C) with a 12 h light / dark cycle (light on: 7:00) with food and water freely available. MAP was purchased from Dainihon Sumitomo Pharma Co. Ltd (Osaka, Japan). SUPERSCRIPT First- Strand Synthesis System for RT-PCR containing reverse transcriptase (RTase) (Superscript II, RNase H-), dithiothreitol (DTT), oligo (dT)12−18 primer, deoxynucleosidetriphosphate (dNTP), RNase H, DNase I and RNase inhibitor were purchased from Invitrogen (San Diego, CA). SV Total RNA Isolation System and PCR Master Mix were from Promega (Madison, WI). DyNAmo SYBER green qPCR Kit were from Finnzymes (Espoo, Finland), DNA 1000 Lab Chips Kit (Agilent Technologies; Palo Alto, CA) were from Takara Bio Co. (Tokyo, Japan). PCR primers were synthesized by Sigma Genosys Japan (Tokyo, Japan). All other chemicals and reagents were purchased from Wako Chemical Co. (Tokyo, Japan) unless otherwise noted.

Acute treatment with MAP

A total of 42 rats were used in this study. In the first experiment, two groups of rats (n=6 each) were used for the behavioral assessment. In the second experiment, six groups of rats (n=5 each) for DBI mRNA analysis were used. Animals were randomly allocated to a control group receiving saline and experimental groups receiving MAP injections. The dose of MAP (5 mg/kg) was selected on the basis of the results obtained previously in several studies carried out in our laboratory [27]. Saline or MAP dissolved in saline was intraperitoneally injected.

The elevated plus-maze test

The elevated plus-maze test [14] is based on the natural aversion of rodents to heights and open-spaces. Thus, subjects prefer to spend time in the closed-space arms rather than in the open-space arms of a maze. Lister [14] has validated the model in rodents with different classes of substances and demonstrated that the elevated plus-maze test is effective for examining anxiogenic-like and anxiolytic-like effects of drugs. The plus-maze apparatus consists of two open-space arms (45×10 cm) and two closed-space arms (45×10 cm, surrounded by 40-cm high walls), with the two pairs of identical platform, which emerged from a central platform (10×10 cm), positioned opposite to each other. The apparatus was elevated 62 cm above the floor [21]. Rats were tested on the maze in randomized order. The test was initiated by placing the rat on the central platform of the maze, facing one of the open-space arms, and letting it move freely. Each session lasted 5 min, being recorded by a video camera. All tests were carried out under dim lighting. Elevated plus-maze tests were performed 0, 1, 2, 4, 8 and 24 h after injections. After each test, the maze was thoroughly cleaned. Behavioral analysis was performed by a trained experimenter who was unaware of treatment of the groups. A couple of classical parameters were collected during the session to assess anxiety, including: (1) open arm duration: the total amount of time the rat spent in the open-space arms; (2) open arm frequency: the frequency of rat entry with all four paws into the open, unprotected arms.

Real time quantitative RT-PCR

The rats were decapitated and the brain was divided into the seven regions; striatum, hippocampus, cerebral cortex, diencephalon, midbrain, pons-medulla, and cerebellum for the DBI gene analysis by RT-PCR. The total RNA was extracted by a method similar to the one described previously [26]. Total RNA (0.5 µg) was incubated with 200 units of the reverse transcriptase in a buffer containing 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP, and 0.5 µg oligo $(dT)_{12\text{-}18}$ primer in the final volume of 20 µl. The mixture was incubated at 42ºC for 50 min, and the reaction was stopped by heating at 70ºC for 15 min. The RNA was removed by adding 2 units of the ribonuclease H to facilitate the synthesis of double stranded cDNA. We used primers specific for DBI mRNA (accession number NM_031853) (upper primer, ACG CTC TGG AAC TTG ATT GC; lower primer, CAG TTG GCT GAG TCT TGA GG; product size, 138 base pairs) and PACAP mRNA (NM_016989) (upper primer, TGT CCG CCA GGA AGT ACC; lower primer, CCG AGT GGC GTT TGG TAA; product size, 105 base pairs). The cDNA was amplified by the quantitative RT-PCR using the DyNAmo SYBER green qPCR Kit (Finnzymes; Espoo, Finland) on the DNA Engine Opticon 2 System (Bio-Rad Laboratories; Hercules, CA) running 40 cycles of the following protocol: 10 min predenaturation at 95ºC, 15 sec annealing at 62ºC for DBI and 61ºC for PACAP, followed by a 20 sec extension at 72ºC. The PCR products were separated by an Agilent 2100 Bioanalyzer (Agilent Technologies; Palo Alto, CA) which utilizes chip-based nucleic acid separation technology. Furthermore, the identification of the amplified PCR products of the DBI and GAPDH cDNAs were determined by the dye terminator cycle sequencing.

Statistical analysis

The primary null hypothesis that was tested was that there is no statistically significant difference in the mean of DBI and PACAP mRNA values when comparing the control group with the MAP group. These results are given as mean with standard error of mean (S.E.M.). A statistical analysis was conducted using the software package SPSS (version 15.0.1, Chicago, IL) and Prism (version 4.0c, GraphPad Software, San Diego, CA) for comparison across the experimental conditions. Statistical comparisons were performed using the Kruskal-Wallis test followed by Dunnett's post-hoc test or repeated two-way analysis of variance

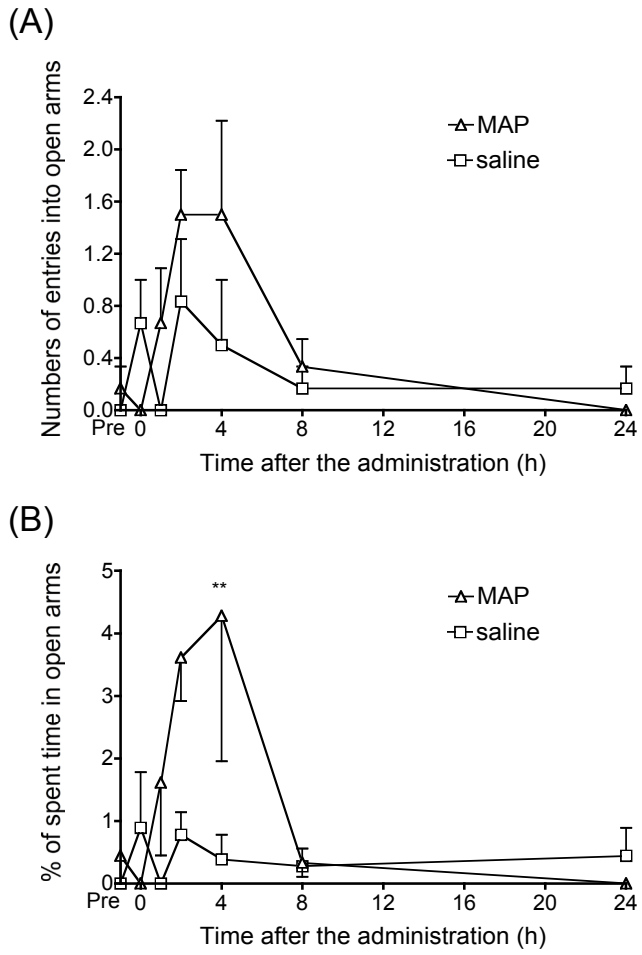

Fig. 1. (A) The frequency of entries in the open-space arms, and (B) the percentages of time spent in the open-space arms at 0, 1, 2, 4, 8, and 24 h after acute administration of MAP (n=6) or saline (n=6). Pre-study was done 30 min prior to the injection. Significantly different from the values between groups by two-way ANOVA test followed by Bonferroni's post-hoc test; $^{**}P<0.01$.

(ANOVA) test followed by Bonferroni's post-hoc test. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

Elevated plus-maze test

Rats acutely treated with MAP (5 mg/kg) showed a significant increase in the time spent in the open-space arms at 4 h after the injection, in comparison with the saline-treated group (Fig. 1).

The mRNA expression of DBI in rat brain after acute administration of MAP

Fig. 2 shows the changes in the expression level of DBI mRNA 2, 4, 8, 16, and 24 h after a single administration of MAP (5 mg/kg). The expression level of DBI mRNA in all the brain regions uniformly decreased 2, 4, 8, and 16 h after the administration of MAP, and then recovered to the expression level of saline-treated group at 24 h. The expression level of DBI mRNA decreased by 24%-54% in the seven brain areas examined: 2 h after the last administration: striatum (38% decrease), hippocampus (39%), cortex (36%), diencephalon (38%), midbrain (34%), pons-medulla (33%), and cerebellum (46%). 4 h after the last administration: striatum (40% increase), hippocampus (29%), cortex (28%), diencephalon (28%), midbrain (37%), pons-medulla (36%), and cerebellum (26%). 8 h after the last administration: striatum (36% increase), hippocampus (34%), cortex (37%), diencephalon (34%), midbrain (40%), pons-medulla (43%), and cerebellum (29%). 16 h after the last administration: striatum (54% increase), hippocampus (33%), cortex (39%), diencephalon (39%), midbrain (38%), pons-medulla (39%), and cerebellum (24%).

The mRNA expression of PACAP in rat brain after acute administration of MAP

Fig. 3 shows the changes in the expression level of PACAP mRNA 2, 4, 8, and 16 h after a single administration of MAP (5 mg/kg). The expression level of PACAP mRNA in all the brain regions uniformly increased 4 and 8 h after the administration of MAP. The expression level of PACAP mRNA increased by 96%-708% in the seven brain areas examined: 4 h after the last administration: striatum (193% increase), hippocampus (96%), cortex (138%), diencephalon (133%), midbrain (111%), pons-medulla (141%), and cerebellum (147%). 8 h after the last administration: striatum (708% increase), hippocampus (183%), cortex

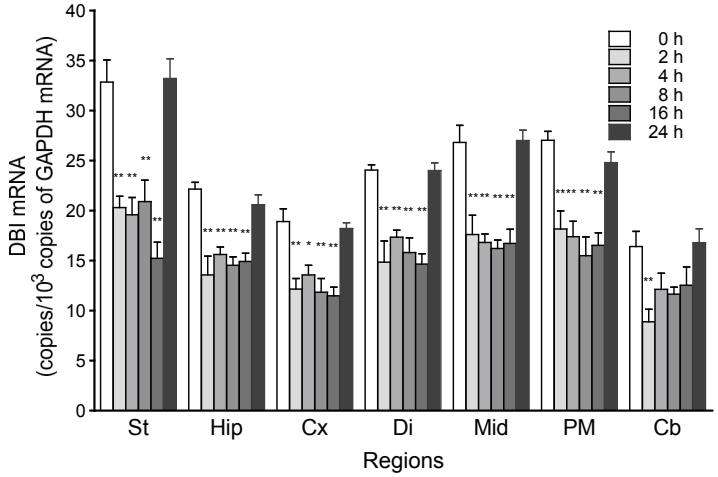

Fig. 2. The expression of diazepam binding inhibitor (DBI) mRNA in seven regions in rat brain 2, 4, 8, 16 and 24 h after acute administration of MAP. The expression level of DBI mRNA was measured by the real-time quantitative RT-PCR. Significantly different from the values of a group treated with saline by Dunnett's post-hoc test following Kruskal-Wallis test; **P*<0.05, ***P*<0.01. St, striatum (saline, n=5; MAP, n=5); Hip, hippocampus (n=5; n=5); Cx, cortex (n=5; n=5); Di, diencephalon (n=5; n=5); Mid, midbrain (n=5; n=5); PM, pons-medulla (n=5; n=5); Cb, cerebellum (n=5; n=5).

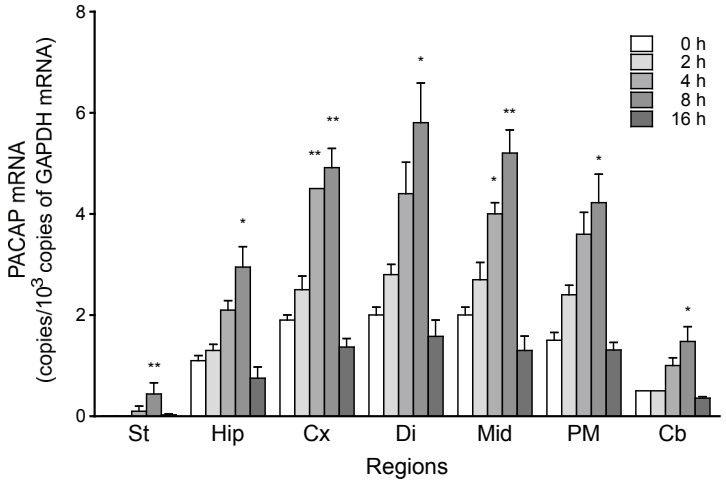

Fig. 3. The expression of pituitary adenylate cyclase-activating polypeptide (PACAP) mRNA in seven regions in rat brain 2, 4, 8, and 16 h after acute administration of MAP. The expression level of DBI mRNA was measured by the real-time quantitative RT-PCR. Significantly different from the values of a group treated with saline by Dunnett's post-hoc test following Kruskal-Wallis test; **P*<0.05, ***P*<0.01. St, striatum (saline, n=5; MAP, n=5); Hip, hippocampus (n=5; n=5); Cx, cortex (n=5; n=5); Di, diencephalon (n=5; n=5); Mid, midbrain (n=5; n=5); PM, pons-medulla (n=5; n=5); Cb, cerebellum (n=5; n=5).

(164%), diencephalon (202%), midbrain (175%), pons-medulla (181%), and cerebellum (259%).

DISCUSSION

We found that the expression of DBI mRNA decreased after a single administration of MAP. This is important because the study showed relations between the anxiolytic behavior and DBI.

In the behavioral assessment (Fig. 1), the elevated plus-maze test in this study shows three phases within 24 h in the group acutely treated with MAP (5 mg/kg) compared to the saline-treated group, that is, acute phase (0-4 h), recovery phase (4-8 h), and stable phase (8-24 h).

In the acute phase (0-4 h), the spent time in the open-space arms significantly increased in the group treated with MAP (5 mg/kg), compared to the saline-treated group, which indicated the anxiolytic effect. This result agrees with the previous study which demonstrated that administration of MAP has no anxiogenic effect in acute phase using the emergence test [4]. Acute exposure of MAP has a psychostimulant (including anti-anxiety) and sympathomimetic effects, whereas chronic exposure of MAP causes the depen-

dence and withdrawal syndrome (including anxiety) [3, 6, 16, 20]. The expression of DBI mRNA decreases in the acute phase (Fig. 2). Based on these results, the significant reduction of the mRNA expression of DBI in all brain areas of rat as well as the relevant anxiolytic behavior after acute administration of MAP agrees with our previous study which exhibited the significant increase of the mRNA expression of DBI in all brain areas of rat after chronic administration of MAP associated with anxiogenesis.

In the recovery phase (4-8 h), the spent time in the open-space arms in the group treated with MAP (5 mg/kg) returns to the level of saline treated group. PACAP mRNA increases during the recovery phase after administration of MAP, (Fig. 3). These results agree with the previous studies as follows: 1) The sequence homologous to cAMP response element (CRE) is found in the 5'-flanking region of the PACAP gene [25]. 2) The administration of amphetamine causes phosphorylation of the cAMP response element binding protein (CREB) and the induction of downstream CREB-regulated gene [22]. Taken together with those results, the administration of MAP can up-regulate the expression of PACAP mRNA in the neurons [24]. 3) The increase in the amount of PACAP enhances the release of DBI from the glial cells into the synaptic cleft mediated through the adenylyl cyclase / protein kinase A (PKA) pathway via PAC1-R in astrocytes [15]. 4) Upon the recovery of the level of DBI peptides in the synaptic cleft, the anxiolytic behavior can be recovered via $GABA_A$ receptor. Although the expression level of DBI mRNA keeps under the control level, the release of DBI in the glial cell to the synaptic cleft can increase.

In the stable phase (8-24 h), the spent time in the open-space arms in the group treated with MAP (5 mg/kg) keeps to the level of saline treated group. The expression level of PACAP mRNA returns to the control. This agrees with the previous studies indicating the negative feedback regulation of the expression level of PACAP mRNA by cAMP as follows: 1) PACAP increases cAMP via PAC1-R [15]. 2) The expression level of PACAP mRNA rapidly decreases in the high level of cAMP signals via folskin [9]. On the other hand, the expression level of DBI mRNA kept under the control level during the stable phase when anxiolysis disappeared. This discrepancy needs to be clarified with further investigation.

In conclusion, the present study for first time elucidated the possible distinct pharmacological property of MAP concerning anxiety, depending on the schedule of the administration, either chronic or acute, and demonstrated that acute administration of MAP decreases significantly the expression level of DBI mRNA in all brain areas of rat, corresponding with the outcome of the behavioral assessment using the elevated plus maze test which showed anxiolytic behavior. These results agree well with our previous study which demonstrated that DBI mRNA increases in several brain areas after chronic administration of MAP, possibly leading to anxiety. Our findings contribute to an understanding of the chemical mechanism of anxiety disorder. Further investigations, however, should be required to clarify the discrepancy findings in this study with neuropharmacological studies.

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