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Acute Cocaine Increases an *N*-methyl-D-aspartate-Dependent Extracellular Signal-Regulated Kinase 1/2 Pathway in Striatal Neurons

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ABSTRACT

Cocaine is an indirect dopamine agonist and upregulates dopamine-mediated glutamatergic transmission in the dorsal striatum. In this study, phosphorylation of *N*-methyl-D-aspartate (NMDA) NR1 subunits, extracellular signal-regulated kinase 1/2 (ERK1/2), and cyclic AMP response element-binding protein (CREB) and expression of c-Fos were simultaneously examined to understand an NMDA-dependent ERK1/2 pathway in striatal neurons of rats treated with acute cocaine. The data demonstrated that intraperitoneal (i.p.) injection of acute cocaine (20 mg/kg) significantly increased the immunoreactivity of phosphorylated (p)NMDA NR1 subunits on serine 896 and 897 in the dorsal striatum. Similarly, pERK1/2, pCREB and c-Fos immunoreactivities also were increased in the dorsal striatum after acute cocaine injection. These data suggest that acute injection of cocaine are necessary for activating NMDA receptors, which in turn stimulates an ERK-1/2 signaling pathway leading to CREB phosphorylation and c-Fos expression in the dorsal striatum.

Key words: MAP kinase, dopamine receptor, psychostimulant

INTRODUCTION

Cocaine is a well known indirect dopamine agonist and upregulates dopamine-mediated glutamatergic transmission in the dorsal striatum. *N*-methyl-D-aspartate (NMDA) receptor is one of the major ionotropic glutamate receptors in the central nervous system. The NMDA subtypes of glutamate receptors are heteromeric ligand-gated ion channels composed of multiple receptor subunits (NR1, NR2A-D and NR3A) (Petrenko et al., 2003). The NMDA receptor complexes may contain two or three different NR1 splice variants (Blahos and Wenthold, 1996; Premkumar and Auerbach, 1997; Dingledine et al., 1999; Stephenson, 2001). Activation of protein kinase C (PKC) and cyclic AMP-dependent protein kinase A (PKA) together leads to the dual phosphorylation of the NR1 subunit serine residues at 896 and 897, respectively (Tingley et al., 1997). Activated NMDA receptors mediate a rapid Ca^{2+} influx and contribute to complicated subsequent intracellular signaling cascades (Impey et al. 1999). For instance, NMDA receptor activation in striatal neurons by the group I metabotropic glutamate receptor (mGluR) agonist 3,5-dihydroxyphenylglycine increased an extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway that resulted in cyclic AMP response element-binding protein (CREB) phosphorylation and *fos* ex-

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pression (Choe and Wang, 2001; Choe et al., 2004).

The present study, therefore, was designed to investigate the hypothesis that the indirect dopamine agonist cocaine upregulates NMDA NR1 phosphorylation that is believed to activate a subsequent intracellular event leading to ERK1/2 and CREB phosphorylation and c-Fos expression in the dorsal striatum.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (200～250 g) were obtained from Hyochang Science Co. (Daegu, Korea). Rats were individually housed in a controlled environment during all experimental treatments. Food and water were provided *ad libitum* and rats were maintained on a 12 hr light/dark cycle. On the day of the experiment injection was made in the quiet room to minimize stress. All animal use procedures were approved by the Institutional Animal Care and Use Committee and were accomplished in accordance with the provisions of the NIH "Guide for the Care and Use of Laboratory Animals".

Experimental design and immunoblotting

The experiment was performed to investigate whether acute cocaine injection alters NMDA NR1 subunit (Ser896, Ser897), ERK1/2 and CREB phosphorylation and c-Fos expression. Rats were randomly divided into 8 groups: acute saline at 0.5, 1, 2 and 4 hr and acute cocaine 0.5, 1, 2 and 4 hr (n =3～4 per group). Each rat received one intraperitoneal (i.p.) injection of acute saline or acute cocaine (20 mg/kg). Throughout the experiments, cocaine (Belgopia, Louvain-La-Neuve, Belgium) was dissolved in physiological saline (0.9% sodium chloride).

For immunoblotting, rats were deeply anesthetized with 8% chloral hydrate (6 ml/kg, i.p.) and decapitated at 0.5, 1, 2 or 4 hr after saline or cocaine injection. Brains were removed, frozen in isopentane at −70°C and stored in a deep freezer. Sections were serially cut in a cryostat and overall dorsal striatum was removed with a steel borer (inner diameter: 2 mm). All tissue samples were lysed in sodium dodecyl sulfate (SDS) sample buffer for 5 min at 95°C. The samples were then sonicated for 30 sec on ice and centrifuged for 10 min at 12,000 g with an Eppendorf tabletop centrifuge. The supernatant fluids were resolved using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked with blocking buffer (2～5% skim milk in TBST). The membrane was probed with each primary antiserum against phosphorylated (p)NR1 (Ser896, 897, 1 : 1,000), NR1 (1 : 1,000), pERK1/2 (1 : 1,000), ERK1/2 (1 : 1,000), pCREB (1 : 500), CREB (1 : 1,000) or c-Fos (1 : 1,000) overnight at 4°C on a shaker. Antiserum for pNR1 (Ser896, 897) and NR1, and c-Fos were purchased from Upstate Biotechnology (Lake Placid, New York, USA) and Oncogene Research Products (San Diego, CA, USA), respectively. Actin antiserum (Sigma-Aldrich) was determined as loading control for c-Fos. Other primary antisera were purchased from Cell Signaling Technology (Beverly, MA, USA). The membrane was then incubated with an appropriate secondary antiserum for 1 hr at room temperature. Unphosphorylated proteins were probed after stripping the same membrane that had been probed for phosphorylated proteins. Immunoreactive protein bands were detected by enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA) on X-ray films.

Quantitation of immunoreactivity (IR)

Immunoreactive protein bands on films were semi-quantified using an imaging digital camera and NIH Image 1.62 software. Briefly, film background was measured and saved as a "blank field" to correct uneven illumination. The upper limit of the density slice option was set to eliminate any background, and this value was used to measure all images. The lower limit was set at the bottom of the LUT scale. The immunoreactive protein bands were measured using a rectangle covered the individual band.

Statistics

Statistical significance of the number of immunoreactive pixels per measured area between groups was determined using a one-way ANOVA on

ranked data followed by a Tukey's HSD (honestly significant difference) test in SAS (Cary, NC, USA). Statistically significant level was taken as $p<0.05$.

RESULTS

This experiment was performed to investigate whether acute cocaine injection (20 mg/kg, i.p.) alters phosphorylation of NMDA NR1 subunits, ERK1/2 and CREB and expression of Fos in the dorsal striatum. Alterations of pNR1-Ser896, pNR1-Ser897, pERK1/2, pCREB and c-Fos IR were examined in the dorsal striatum at 0.5, 1, 2 or 4 hr after acute saline or cocaine injection. The results showed that acute cocaine injection in the dorsal striatum significantly increased both pNR1 IR at all the time point except for 4 hr at pNR1-Ser897 as compared to acute saline injection (Fig. 1). P-NR1-Ser896 IR was significantly altered at 0.5 hr, reached a peak at 2 hr and sharply decreased at 4 hr after acute cocaine injection, whereas pNR1-Ser897 IR was significantly altered at 0.5 hr, prolonged by 2 hr and get back to the basal levels at 4 hr. Parallel to the pNR1-Ser896 and pNR1-Ser897 induction, acute cocaine injection also sig-

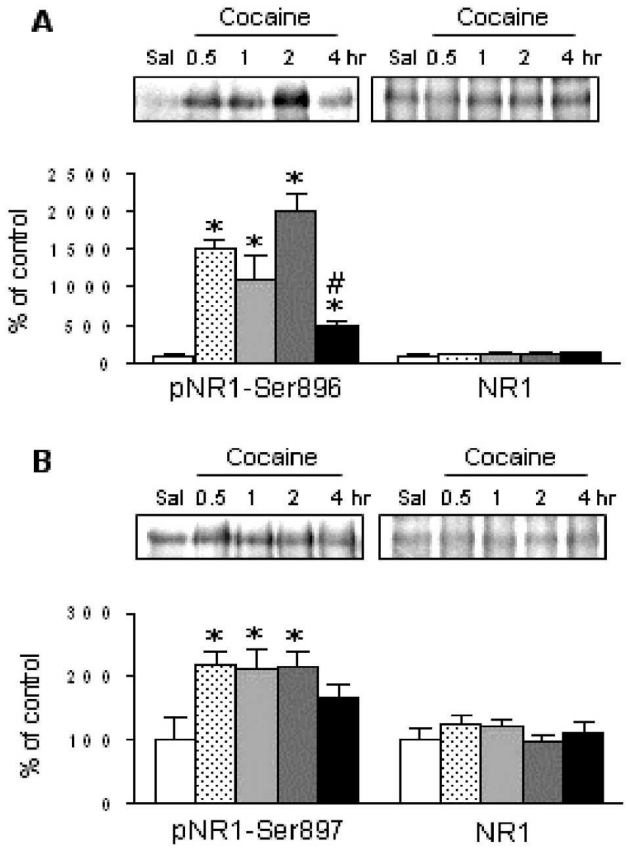

Fig. 1. Western immunoblot analysis for the effect of i.p. injection of acute cocaine injection on pNR1-Ser896 and NR1 (A) and pNR1-Ser897 and NR1 (B) induction in striatal neurons at 4 different time points. Semi-quantitative analysis confirms that acute cocaine injection caused an increase in the IR of pNR1 in striatal neurons (n=4 per group). *$p<0.05$ vs. acute saline; #$p<0.05$ vs. acute cocaine at 2 hr time point.

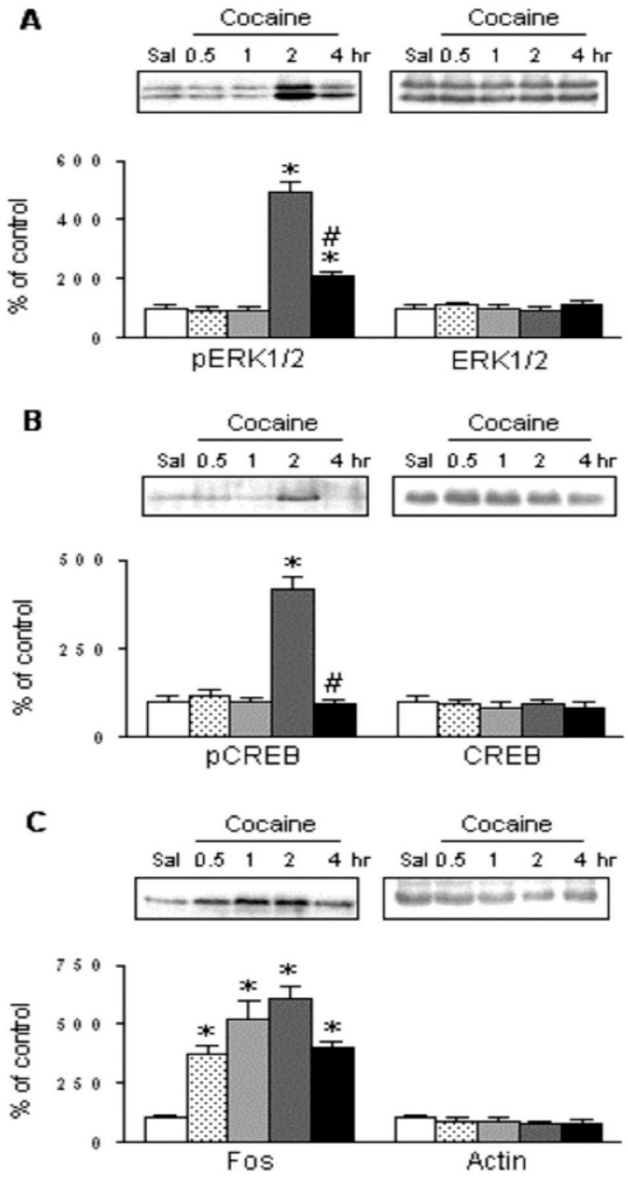

Fig. 2. Western immunoblot analysis for the effect of i.p. injection of acute cocaine injection on pERK1/2 and ERK1/2 (A), pCREB and CREB (B), and c-Fos and actin (C) induction in striatal neurons at 4 different time points. Semi-quantitative analysis confirms that acute cocaine injection caused an increase in the IR of pERK1/2, pCREB and Fos in striatal neurons (n=4 per group). *$p<0.05$ vs. acute saline; #$p<0.05$ vs. acute cocaine at 2 hr time point.

nificantly increased pERK1/2 and pCREB IR at 2 hr (Fig. 2A, B), whereas c-Fos IR (Fig. 2C) was significantly increased at all time point. However unphosphorylated NR1, ERK1/2 and CREB and actin IR was not altered throughout the experiment.

DISCUSSION

Previous study from Choe and McGinty (2001) demonstrated that the cAMP analog Sp-8-Br-cAMP facilitated NMDA receptor phosphorylation and an ERK1/2 signaling cascade leading to CREB phosphorylation in the striatum *in vivo*. It was therefore hypothesized that acute cocaine injection activates NMDA receptors and a subsequent ERK1/2 pathway leading to pCREB and Fos expression in striatal neurons. The present data showed that acute cocaine administration in the dorsal striatum significantly increased NMDA NR1 subunit, ERK1/2 and CREB phosphorylation and c-Fos expression. These data suggest that acute cocaine injection activates NMDA NR1 via D1 receptor-mediated intracellular mechanisms since glutamate levels were altered by acute injection of cocaine in our previous study (Shin et al., 2007).

Numerous studies demonstrated that D1 dopamine receptors are involved in NMDA NR1 phosphorylation (Choe and McGinty, 2000). Cocaine upregulates adenylate cyclase/cAMP cascades leading to cAMP dependent- or independent-PKA activation via excitatory G-protein coupled D1 receptors in neurons (Walsh et al., 1968; White and Kalivas, 1998). Activated PKA causes NMDA NR1 phosphorylation (Tingley et al., 1997; Dudman et al., 2003), which potently activates mitogen-activated protein (MAP) kinase via increased extracellular and internal Ca^{2+} releases (York et al., 1998; Impey et al., 1999). ERK1/2 has been found to be an effective kinase for CREB phosphorylation. For instance, ERK1/2 cascades couple CREB phosphorylation in the CA1 area of the hippocampus (Roberson et al., 1999), pheochromocytoma 12 cells and cultured hippocampal neurons (Impey et al., 1998). ERK1/2 is also found to mediate glutamate-induced phosphorylation of the transcription factors such as CREB and Elk-1 in the striatum (Sgambato et al., 1998; Davis et al., 2000; Choe and McGinty, 2001). The fact that pERK1/2 induction in the dorsal striatum by acute cocaine injection was tremendously increased as compared with acute saline, suggesting that D1-coupled intracellular mechanisms are involved in cocaine injection as demonstrated by extensive previous studies for last decades. This prediction is supported by the finding that repeated cocaine increased phosphorylation of NMDA NR1 subunits on serine 896 and 897, ERK1/2 and CREB, which is significantly decreased by D1 or NMDA antagonism.

In summary, this study provides evidence that acute cocaine injection facilitated extracellular dopamine release in the dorsal striatum. In addition, cocaine increased phosphorylation of NMDA NR1 subunits on serine 896 and 897, ERK1/2 and CREB and expression of c-Fos. These findings suggest that acute cocaine is capable to increase dopamine, which in turn regulates an NMDA-associated ERK1/2 pathway via a D1-dependent mechanism that integrates the effects of cocaine in striatal projection neurons. These data may provide helpful information to understand the role of acute cocaine in the regulation of NMDA-associated cellular events leading to c-Fos expression in the dorsal striatum.

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