Repeated cocaine administration increases N-methyl-D-aspartate NR1 subunit, extracellular signal-regulated kinase and cyclic AMP response element-binding protein phosphorylation and glutamate release in the rat dorsal striatum

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A B S T R A C T

This study was conducted to determine the phosphorylation state of N-methyl-D-aspartate (NMDA) NR1 subunit on serine residues 896 (Ser896) and 897 (Ser897), the extracellular signal-regulated kinase 1/2 (ERK1/2), and the cyclic AMP response element-binding protein (CREB) after repeated exposure to cocaine (20 mg/kg, once daily for 9 days) in the dorsal striatum of rats. The real-time changes of glutamate concentration evoked by repeated cocaine injections were examined using a glutamate biosensor in order to evaluate the correlation between glutamate concentration and the change in these phosphoproteins. The results of this study showed that the immunoreactivity of phosphorylated (p)NMDA NR1 subunit at Ser896 and Ser897 as well as pERK1/2, but not pCREB, in the dorsal striatum was increased at 30 min and then returned to basal levels 4 h after repeated cocaine injections. Similarly, glutamate responses evoked by repeated cocaine injections were also increased 30 min after repeated cocaine injections for 3 days and were prolonged by the 9th day of treatment. However, the glutamate responses were not detected at 4 h after repeated cocaine injections for 5 days. In addition, the elevated immunoreactivity of the phosphoproteins 2 h after repeated cocaine injections was attenuated by the blockade of dopamine D1 receptors and NMDA receptors with the SCH23390 or MK801 antagonists, respectively. These findings suggest that glutamate release and dopamine D1 and NMDA receptor stimulation after repeated exposure to cocaine are associated with NMDA NR1 subunit, ERK1/2 and CREB phosphorylation in the dorsal striatum.

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1. Introduction

Cocaine is an indirect dopamine agonist and repeated exposure to cocaine upregulates the release of glutamate in the dorsal striatum (Shin et al., 2007). The increased levels of glutamate after repeated cocaine administration activate N-methyl-D-aspartate (NMDA) receptors which play an important role in the regulation of the Ca2+-dependent protein kinases, such as extracellular signal-regulated kinase 1/2 (ERK1/2) in the dorsal striatum (Jiao et al., 2007). The NMDA receptor is one of the major ionotropic glutamate receptors of the central nervous system. The NMDA subtypes of glutamate receptors are heteromeric ligand-gated ion channels composed of multiple receptor subunits, such as NR1, NR2A-D and NR3A (Petrenko et al., 2003). It has been shown that the NMDA receptor complexes contain two or three different NR1 splice variants (Blahos and Wenthold, 1996; Dingledine et al., 1999; Stephenson, 2001). The combined activation of protein kinase C (PKC) and cyclic AMP-dependent protein kinase A (PKA) leads to the dual phosphorylation of the NR1 subunit at serine residues 896 and 897, respectively (Tingley et al., 1997). The activation of NMDA receptors mediates a rapid Ca2+ influx and contributes to the complicated subsequent intracellular signaling cascades (Impy et al., 1999). For instance, NMDA receptor activation in striatal neurons by the group I metabotropic glutamate receptor (mGluR) agonist 3,5-dihydroxyphenylglycine increases ERK1/2 signaling pathways that result in the phosphorylation of the cyclic AMP response element-binding protein (CREB) (Choe and Wang, 2001; Choe et al., 2004).

A biotechnology-based glutamate microbiosensor was developed by our research group and was effectively used for the real-time and immediate measurements of altered glutamate concentration in vitro as well as in vivo (Rahman et al., 2005), although microdialysis has been widely performed to determine the changes in glutamate release in the brain evoked by addictive drugs, such as cocaine (Williams and Steketee, 2004). Using the glutamate microbiosensor, we recently demonstrated that repeated, but not acute, injections of cocaine increase the release of extracellular glutamate in the dorsal striatum (Shin et al., 2007), demonstrating that the glutamate biosensor is an effective tool for monitoring the real-time changes of glutamate concentration in response to stimulant drug exposure.

The present study was therefore designed to investigate how repeated cocaine injections regulate NMDA NR1 phosphorylation,
which is believed to activate subsequent intracellular events that result in ERK1/2 and CREB phosphorylation. The changes in glutamate release evoked by repeated cocaine administration along the time course were also determined by the real-time biosensing of glutamate. This approach allows us to evaluate the correlation between glutamate concentration and the changes in these phosphoproteins within the dorsal striatum.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats (200–250 g) were obtained from Hyochang Science Co. (Daegu, Korea). The rats were individually housed in a controlled environment during all experimental treatments. Food and water were provided ad libitum, and the rats were kept on a 12 h light/dark cycle. On the day of the experiment, injections were made in a home cage in a quiet room in order to minimize stress to the animals. All animal use procedures were approved by the Institutional Animal Care and Use Committee and were carried out in accordance with the provisions of the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

The dopamine D1 receptor antagonist (R)-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH23390, 7.5 nmol) (Tocris Cookson, Ellisville, MO, USA) or the NMDA antagonist (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK801, 2 nmol) (Tocris Cookson) was dissolved in dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO, USA) and then diluted in artificial cerebrospinal fluid (aCSF) (123 mM NaCl, 0.86 mM CaCl2, 3.0 mM KCl, 0.89 mM MgCl2, 0.50 mM NaH2PO4 and 0.25 mM Na2HPO4 aerated with 95% O2/5% CO2, pH 7.2–7.4). The drug concentrations used were determined in previous studies (Wang and McGinty, 1995; Choe and Wang, 2002; Ahn et al., 2007). Cocaine (Belgophia, Louvain-La-Neuve, Belgium) was dissolved in physiological saline (0.9% sodium chloride, NaCl) and each rat received repeated intraperitoneal injections of cocaine (20 mg/kg) once daily for 9 consecutive days. In addition to each drug injection, DMSO/aCSF or NaCl was injected into the center of the dorsal striatum or the peritoneum, as a control, 5 min before the final injection of cocaine or saline in each experiment.

2.3. Surgery and intrastral injection

The rats were anesthetized with 8% chloral hydrate (5.8 ml/kg) and placed in a Kopf stereotactic apparatus. Under aseptic conditions, a 23-gauge stainless steel guide cannula (inner diameter: 0.29 mm, 10 mm in length) was implanted at the coordinates of 1 mm anterior to the bregma, 2.5 mm to the right of the midline, and 4 mm below the surface of the skull. The guide cannula was sealed with a stainless steel wire of the same length (10 mm). The rats were allowed 5 days to recover from surgery. On the day of the experiment, the inner steel wire was replaced by a 30-gauge stainless steel injection cannula (inner diameter: 0.15 mm) with a length of 12.5 mm that protruded 2.5 mm beyond the guide cannula. Through the injection cannula, SCH23390, MK801 or vehicle was infused unilaterally into the central part of the right dorsal striatum in a volume of 1 µl at a rate of 0.2 µl/min in freely moving rats because the compounds may not penetrate blood brain barrier in the brain. The injection progress was monitored by observing the movement of a small air bubble through the length of precalibrated PE-10 tubing inserted between the injection cannula and a 10 µl Hamilton microsyringe. After the completion of the injection, the injector was left in place for an additional 5 min to reduce any possible backflow of the solution along the injection tract.

2.4. Western immunoblotting

The rats were deeply anesthetized with 8% chloral hydrate and decapitated at 0.5, 1, 2 or 4 h after the final injection of repeated saline or cocaine. The brains were removed, frozen in isopentane at −70 °C and stored in a deep freezer. Sections were serially cut in a cryostat and the entire dorsal striatum was removed with a steel borer (inner diameter: 2 mm) as described previously (Shin et al., 2007). All tissue samples were transferred to homogenization buffer (10 mM pH 7.4 Tris–HCl, 5 mM NaF, 1 mM Na3VO4, 1 mM EDTA, 1 mM EGTA), sonicated for 30 s on ice, and centrifuged at 13,000 rpm for 30 min at 4 °C. The pellet containing mainly nuclei and large debris was discarded, and the supernatant was centrifuged again at 13,000 rpm for 30 min at 4 °C. The supernatant was resolved using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were 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 (1: 1000), pNR1-Ser897 (1: 1000), NR1 (1: 1000), pERK1/2 (1: 1000), ERK1/2 (1: 1000), pCREB (1: 500) or CREB (1: 1000) overnight at 4 °C on a shaker. Antiserum for pNR1-Ser896, pNR1-Ser897 or NR1 was purchased from Upstate Biotechnology (Lake Placid, New York, USA). Other primary antisera were purchased from Cell Signaling Technology (Beverly, MA, USA). The membrane was then incubated with an appropriate secondary antiserum for 1 h at room temperature. Unphosphorylated proteins were probed for stripping the same membrane that was previously probed for phosphorylated proteins. Immunoreactive protein bands were detected using enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA) on X-ray films.

2.5. Immunohistochemistry

As in the Western blot analysis, rats were deeply anesthetized with 8% chloral hydrate and transcardially perfused with 4% paraformaldehyde at 4 °C. The brains were removed and post-fixed in 10% sucrose/4% paraformaldehyde for 2 h at 4 °C and then placed in 20% sucrose/phosphate-buffered saline (PBS) at 4 °C overnight. Using a sliding microtome, 40 µm frozen sections were cut. Two sections per antiserum per brain were collected at the striatal levels and processed for immunohistochemistry according to the method described previously (Choe et al., 2004). Briefly, sections were incubated with the primary antiserum for 20 h at 4 °C on a shaker. Sections were then incubated in goat anti-rabbit secondary antiserum (Vector Laboratories, Burlingame, CA, USA) for 1 h, followed by avidin–biotin–peroxidase reagents (Elite Vectastain kit, Vector Laboratories) for 1 h at room temperature. Diaminobenzidine was used as the chromagen and NiCl2 was added to enhance the reaction product.

2.6. Quantitation of immunoreactivity

Immunoreactive protein bands on the films were semi-quantified using an imaging digital camera and NIH Image 1.62 software. Briefly, the film background was measured and saved as a “blank field” to correct for 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 that encompassed the individual band.

2.7. Preparation of glutamate microbiosensor

The glutamate microbiosensor was prepared as described previously (Rahman et al., 2005). Briefly, a micropipette was made by pulling a glass capillary (inner diameter 1.1–1.2 mm, length 75 mm) (Chase Scientific Glass Inc., Rockwood, TN, USA) with a microelectrode.
The tip of the micropipette was broken to the desired inner diameter (150 μm) with a diamond cutter. A Pt-wire (Pt 99.999%, 100 μm in diameter) (Johnson Matthey Inc., West Chester, PA, USA) of about 2 mm in length was connected to a copper wire for electrical contact using electroconductive silver paste (Dotite Electroconductive, Type D-500) (Fujikura Kasei Co., Japan). The copper wire-connected Pt electrode was introduced into the micropipette and advanced until it protruded about 0.2–0.3 mm from the hollow opening of the micropipette. The tip of the micropipette was then heated gently in a butane flame until the glass collapsed and sealed the Pt electrode. The other end of the micropipette was fixed using epoxy resin. The total length of the Pt microelectrode was 40 mm. The Pt microelectrode was cleaned by cycling the potential between +1.6 and −0.16 several times in a 1 M H2SO4 solution, followed by washing three to four times with distilled water and then used in all subsequent experiments. The resulting Pt microelectrode was coated with a conductive polymer (CP) through the electropolymerization of a 5,2′-5′,2″-terthiophene-3′-containing carboxylic acid monomer (Lee and Shim, 2001) by cycling the potential three times at a fast scan rate. The CP-coated Pt microelectrode was then immersed in a 0.1 M PBS containing 10 mM 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide (EDC) for 6 h in order to activate the carboxylic acid groups of the CP. Glutamate oxidase (GluOx, EC. 1.4.3.11, 10.8 units mg−1) (Sigma-Aldrich, St. Louis, MO, USA) and ascorbate oxidase (AsOx, EC. 1.10.3.3, 100 units mg−1) (Sigma-Aldrich) were co-immobilized onto the CP layer through the formation of covalent bonds between the carboxylic acid groups of the CP and the amine groups of GlOx and AsOx. The GlOx/CP modified Pt microelectrode was used as a working electrode for the detection of glutamate as described previously (Shin et al., 2007). Briefly, amperometric responses were recorded using a Potentiostat (Kosentech model KST-P2, Korea) with two electrode configurations: a glutamate microbiosensor and a micro-Ag/AgCl electrode as the working and reference/counter electrodes, respectively. The experiment was carried out by applying a potential of +0.40 V at the GlOx/CP/Pt microbiosensor to oxidize the H2O2 generated from the enzymatic reaction. All the microbiosensors were calibrated before and after measurements as shown in Fig. 4.

### 2.8. Glutamate detection with a microbiosensor

Five minutes before each time point, the rats were anesthetized with 8% chloral hydrate and placed in a Stoelting stereotaxic apparatus. Under aseptic conditions, the enzyme-coated electrode and the reference electrode of the glutamate microbiosensor were implanted at the coordinates of 1 mm anterior to the bregma, 2.5 mm to the right of the midline and 4 mm below the surface of the dura. The sensor tip was unilaterally inserted into the central part of the right dorsal striatum. The output of the electrode was connected to the electrochemical detector Potentiostat. The electrochemical signal was read and stored by the workstation. The glutamate responses in the dorsal striatum can be measured immediately after the implantation of the microbiosensor electrodes. Before the measurement, one sensor from the same batch was calibrated with a series of glutamate standard solutions. In this study, 16 representative time points (before repeated saline or cocaine, and repeated saline or cocaine at 0.5, 1, 2 and 4 h) were selected based on the results of previous studies showing that glutamate levels were significantly increased at 30 min after repeated cocaine injections, as compared to the levels after repeated saline injections (Rahman et al., 2005; Shin et al., 2007).

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**Fig. 1.** The immunoreactivity of pNR1-Ser896 (A) pNR1-Ser897 (B) and pERK1/2 (C) in the dorsal striatum was increased 0.5 h after repeated cocaine injections, whereas pCREB immunoreactivity was delayed (D) (n=4 per group). *P<0.05 vs. repeated saline injections; †P<0.05 vs. repeated cocaine injections at the 2 h time point.

**Fig. 2.** The immunoreactivity of pNR1-Ser896 and Ser897 as well as pCREB induced by 2 h after the final injection of repeated saline (A, B, C) or repeated cocaine (D, E, F) injections was elevated in the cytoplasmic (pNR1) and nuclear (pCREB) compartments of striatal neurons. Bar represents 100 μm.
2.9. Statistics

The statistical significance of glutamate concentrations and the number of immunoreactive pixels per measured area between groups was determined using one-way ANOVA on ranked data, followed by a Tukey's HSD (honestly significant difference) test in SAS (Cary, NC, USA). The level of statistical significance was set at $P<0.05$.

3. Results

3.1. Repeated cocaine injections increased the immunoreactivity of pNR1-Ser896 and Ser897, pERK1/2, and pCREB in the dorsal striatum

This experiment was conducted to determine whether repeated cocaine injections (20 mg/kg), once daily for 9 days, alter the phosphorylation of NMDA NR1 subunit, ERK1/2, or CREB in the dorsal striatum. The alterations of pNR1-Ser896, pNR1-Ser897, pERK1/2, or pCREB immunoreactivity were examined in the dorsal striatum at 0.5, 1, 2 or 4 h after repeated saline or cocaine injections. The results showed that repeated cocaine injections increased the immunoreactivity of pNR1-Ser896 and Ser897 at all of the time points when compared to that in rats subjected to repeated saline injections (Fig. 1A, B). The immunoreactivity of pNR1-Ser896 and Ser897 was altered at 0.5 h, peaked at 1 or 2 h and sharply decreased at 4 h after repeated cocaine injections. The repeated cocaine injections also increased pERK1/2 immunoreactivity at all of the time points (Fig. 1C), but pCREB immunoreactivity (Fig. 1D) was increased only 2 h after repeated cocaine injections. The intensity of pNR1-Ser896 immunoreactivity after repeated cocaine injections was increased within the cytoplasmic compartment when compared to that seen in rats treated with repeated saline injections (Fig. 2A, B, D, E). In contrast, pCREB immunoreactivity was increased within the nuclear compartment after repeated cocaine injections when compared with the rats subjected to repeated saline injections (Fig. 2C, F).

3.2. Blockade of dopamine D1 receptors and NMDA receptors decreased the immunoreactivity of pNR1-Ser896 and Ser897, pERK1/2, and pCREB after repeated cocaine injections in the dorsal striatum

Since repeated cocaine injections increased the immunoreactivity of pNR1-Ser896 and Ser897, ERK1/2 and CREB in the dorsal striatum, the involvement of dopamine D1 receptors or NMDA receptors in the regulation of the phosphoproteins was investigated by either the intraperitoneal injection of the D1 antagonist SCH23390 (0.1 mg/kg) or the intrastriatal infusion of the NMDA antagonist MK801 (2 nmol) 5 min before the final injection of repeated saline or cocaine injections. The results demonstrated that SCH23390 or MK801 decreased the immunoreactivity of the phosphoproteins that were elevated by repeated cocaine injections in the dorsal striatum (Fig. 3). The doses of the antagonists were determined from the results of previous studies (Choe and Wang, 2002; Shin et al., 2007; Wang and McGinty, 1995).

3.3. Repeated cocaine injections increased glutamate responses in the dorsal striatum

Parallel to the change of the immunoreactivity of the phosphoproteins, the change of glutamate release after repeated cocaine injections along the time course was examined using real-time glutamate biosensing in the dorsal striatum. The glutamate concentrations were calculated with the calibration curves obtained before and after the each experiment (Fig. 4), and the real-time measurements of the change in glutamate responses after repeated cocaine injections in the dorsal striatum were shown in Fig. 5. The glutamate concentrations in the dorsal striatum before repeated saline or cocaine

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Fig. 3. The blockade of dopamine D$_1$ receptors and NMDA receptors with SCH23390 (0.1 mg/kg) and MK801 (2 nmol), respectively, decreased the immunoreactivity of pNR1-Ser896 (A), pNR1-Ser897 (B), pERK1/2 (C) and pCREB (D) that was previously elevated by repeated cocaine injections in the dorsal striatum ($n=3$–$4$ per group). Veh, vehicle; Sal, saline; SCH, SCH23390; MK, MK801. *$P<0.05$ vs. vehicle + repeated saline injections; †$P<0.05$ vs. vehicle + repeated cocaine injections at 2 h time point.

Fig. 4. Glutamate response–time plots obtained by the addition of varying amounts of standard glutamate solutions (0.2, 0.4, 0.9, 1.6, 2.0, 4.0, 6.0, 8.0 and 10.0 µM) in 0.1 M PBS at pH 7.4 (A). Calibration plots of current vs. glutamate concentration detected by the glutamate microbiosensor (B).
injections and after repeated saline injections were not altered at any time point. However, the glutamate concentrations in the dorsal striatum at 0.5 and 1 h after repeated cocaine injections were altered at day 3, sharply increased at day 7 and peaked at day 8 as compared to the glutamate levels after repeated saline injections (Fig. 5C, G, H). The changes in glutamate concentrations in the dorsal striatum at 2 and 4 h after repeated cocaine injections were altered at day 3 and then decreased at day 7 and day 5, respectively (Fig. 5C, E, G). Throughout the data, the amount of change in glutamate responses was much greater at 0.5 h after repeated cocaine injections than at the 1, 2, and 4 h time points.

4. Discussion

The results of this study showed that repeated cocaine injections increased the immunoreactivity of NMDA NR1 subunit on Ser896 and Ser897 in the dorsal striatum. The increased immunoreactivity of the NMDA NR1 subunit after repeated cocaine injections was attenuated...
by the blockade of dopamine D1 receptors and NMDA receptors using the SCH23390 and MK801 antagonists, respectively. These data suggest that dopamine D1 receptor stimulation in response to the repeated cocaine administration is responsible for phosphorylating NMDA NR1 subunit in the dorsal striatum. It has been known that cocaine administration facilitates NMDA receptor phosphorylation via dopamine D1 receptor-coupled PKA activation. Activated PKA or PKC then induces NMDA NR1 phosphorylation (Dudman et al., 2003; Tingley et al., 1997), which potently activates MAPK via the increased release of extracellular and internal Ca2+ (Impey et al., 1999; York et al., 1998). Thus, combined activation of PKA and PKC may lead to the dual phosphorylation of the NR1 subunit at serine residues 896 and 897 in the dorsal striatum.

Similarly, the results of this study showed that the increased amount of pERK1/2 and pCREB immunoreactivity in the dorsal striatum after repeated cocaine injections was attenuated by the pretreatment with either dopamine D1 receptor or NMDA receptor antagonist, suggesting that dopamine D1 receptor or NMDA receptor stimulation is required for the phosphorylation of ERK1/2 and CREB after repeated cocaine administration. The ERK1/2 has been found to be an effective kinase for CREB phosphorylation in the dorsal striatum and the CA1 area of the hippocampus (Choe and McGinty, 2001; Roberson et al., 1999). The ERK1/2 was also found to mediate glutamate-induced phosphorylation of transcription factors, such as CREB and Elk-1 in the striatum (Choe and McGinty, 2001; Davis et al., 2000; Sgambato et al., 1998). These findings suggest that dopamine D1- and glutamate-dependent signaling cascades leading to ERK/12 and CREB phosphorylation in the dorsal striatum are induced by NMDA NR1 phosphorylation after repeated cocaine administration. Taken together these data suggest that the dual activation of dopamine D1 receptors and NMDA receptors after repeated cocaine injections closely regulates ERK1/2 and CREB phosphorylation via NMDA NR1 phosphorylation in the dorsal striatum. However, possible mechanisms underlying delayed response on the pCREB immunoreactivity as compared with prompt response on the NMDA NR1 and pERK1/2 immunoreactivity would be investigated in future studies.

The results of this study also demonstrated that the real-time changes of glutamate responses detected by the microbiosensor were significantly elevated from day 3 to day 9 after repeated cocaine injections, however these responses were not altered by repeated saline injections. The results of this study also showed that the elevated glutamate levels in the dorsal striatum at 2 and 4 h after repeated cocaine injections were significantly attenuated on day 7 as compared with the 0.5 and 1 h time points on day 7. These data suggest that elevated glutamate levels only exist in the dorsal striatum for 1 h and are then degraded, even after repeated exposure to cocaine. Regardless of the kinetics of glutamate degradation, the effect of glutamate on striatal neurons seems to be prolonged for a period of time, because delayed immunoreactivity of the NMDA NR1 subunit in the dorsal striatum. Therefore, the application of real-time biosensing in the detection of the changes of glutamate release could be helpful in predicting the temporal changes of cellular events in response to repeated exposure to cocaine. However, the precise mechanisms underlying the correlation between the release of glutamate and subsequent signaling cascades should be investigated in depth.

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