Cocaine regulates ezrin-radixin-moesin proteins and RhoA signaling in the nucleus accumbens

Wha Young Kim

Department of Medical Science

The Graduate School, Yonsei University

Cocaine regulates ezrin-radixin-moesin proteins and RhoA signaling in the nucleus accumbens

Directed by Professor Jeong-Hoon Kim

The Doctoral Dissertation submitted to the Department of Medical Science, The Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Wha Young Kim

December 2009

This certifies that the Doctoral Dissertation of Wha Young Kim is approved.

Thesis Supervisor: Jeong-Hoon Kim

Thesis Committee Member: Dong Goo Kim

Thesis Committee Member: Chan Hyung Kim

Thesis Committee Member: Bae Hwan Lee

Thesis Committee Member: Won Taek Lee

The Graduate School

Yonsei University

December 2009

ACKNOWLEDGEMENT

First of all, I would like to give my deepest gratitude to my thesis advisor**,** Dr. Jeong-Hoon Kim, for his continuous support and insightful advice. He gave me the right direction when I was lost and encouragement when I had difficulty with experiments. Most of all, he showed me how life in a research laboratory can be enjoyable. I would also like to thank my dissertation committee, Dr. Dong Goo Kim, Dr. Chan Hyung Kim, Dr. Bae Hwan Lee, and Dr. Won Taek Lee, for their guidance and comments to make my thesis better.

I owe many thanks to the members of the Kim's Drug Addiction Laboratory, Dr. Seung woo Kim, Ju Kyong Jang, Hyung Shin Yoon, So Ra Shin, Jung Keun Shin, and Hye Kyoung Park, for their assistance, encouragement, and friendship. I especially thank Dr. Soo Kyung Choi for her warm encouragement and friendship. I thank all members of Department of Physiology, Yonsei University College of Medicine, as well.

I also owe a special debt of gratitude to my parents. They

showed deep patience and provided incessant love throughout this project. I would also like to express my deep gratitude to my husband, Eui Ho Park, for his support and encouragement. Most of all, I thank Jesus Christ for the love and guidance he has bestowed upon me.

Table of contents

List of figures

ABSTRACT

Cocaine regulates ezrin-radixin-moesin proteins and RhoA signaling in the nucleus accumbens

Wha Young Kim

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Jeong-Hoon Kim)

Cocaine induces structural plasticity in the nucleus accumbens (NAcc), which may importantly contribute to the development of drug addiction. The ezrin-radixin-moesin (ERM) proteins have been implicated in cell-shape determination by crosslinking F-actin to plasma membranes. However, it has not been determined yet how ERM proteins are regulated by cocaine. Here we show that the phosphorylation levels of ERM protein are dose- and time-dependently decreased in the NAcc by a single injection of cocaine (15 or 30 mg/kg, i.p.). Further, we show that not only the amount of active RhoA, a small

GTPase protein, but also the membrane to cytosol ratio of RhoA is significantly reduced in the NAcc by cocaine (15 or 30 mg/kg, I.P.), while the phosphorylation levels of ERM protein are also decreased by bilateral microinjections in this site of the Rho kinase inhibitors, Y27632 (1.0 or 10.0 μg/0.5 μl/side) or RKI II (0.5 or 2.0 μg/0.5 μl/side). Together, these results suggest that cocaine reduces phosphorylated ERM levels in the NAcc by making down-regulation of RhoA-Rho kinase signaling, which may importantly contribute to initiate synaptic morphological changes in the NAcc leading to drug addiction.

Key words : cocaine; ERM; RhoA; nucleus accumbens; structural plasticity; addiction

Cocaine regulates ezrin-radixin-moesin proteins and RhoA signaling in the nucleus accumbens

Wha Young Kim

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Jeong-Hoon Kim)

I. INTRODUCTION

Drug addiction is a form of chronic brain disease that is characterized by compulsive drug seeking and taking.[1] It is known to be mediated by rewarding circuit consisted of several brain regions including ventral tegmental area (VTA), nucleus accumbens (NAcc), and prefrontal cortex (PFC).[2] Among them, the NAcc is known to play an important role mediating the expression of both acute and chronic effects of drugs of abuse. It has been shown that cocaine produces the structural changes in this site by increasing dendritic spine density

after developments of behavioral sensitization and self-administration.[3-6] Further, the NAcc has more recently been shown to increase its dendritic spine density even after acute cocaine injection, although appeared in different time-scale compared to chronic cocaine-treated rats.[7] These results suggest that cocaine induces structural plasticity in the NAcc and thereby it may eventually lead to the development of addictive behaviors. However, relevant biochemical processes which mediate this structural plasticity are not much known yet. Thus, in the present study it was sought to determine whether a single acute cocaine injection may elicit a form of metaplasticity, defined as a change in the biochemical state of synapses that alters their ability to generate synaptic plasticity,[8] which may contribute to a sequence of changes eventually leading to the structural changes of the NAcc.

The ezrin-radixin-moesin (ERM) proteins (about 75 KDa in molecular weight) have been implicated in cell-shape determination and share a high degree of homology among themselves.[9, 10] They contain a filamentous F-actin binding site at C-terminal regulatory domain and thereby crosslink it to plasma membrane. Thus, ERM proteins have been suggested to function as cross-linkers between actin filaments and plasma membranes, and to be involved in microvilli formation, cell adhesion, cell motility, cytokinesis, and the integration of membrane transport with signaling pathways.[9] In order to stably maintain F-

actin binding and consequently link to the plasma membrane, ERM proteins require a threonine residue in this domain being phosphorylated. By modulating this phosphorylation levels, ERM proteins contribute to the cycling of actin cytoskeleton in between globular (G) and filamentous (F) forms, and play an important role during development and regeneration of neurons growing in a process controlled by extrinsic factors and cytoskeletal interactions. The suppression of ERM proteins was found to alter growth cone morphology, motility, and process formation in primary cultured neurons.[11] Further, interestingly, it has been shown that ERM proteins regulate dendritic spine morphology[12] and that the levels of F-actin were increased in the NAcc by acute cocaine injection in saline pre-exposed, while they were decreased in cocaine pre-exposed rats.[7, 13] Although these results clearly indicate that cocaine regulates F-actin levels in the NAcc, whether ERM proteins have a role in this process has remained unresolved.

ERM phosphorylation on the C-terminal threonine requires activity of the small GTP-binding protein RhoA and its down-stream effector Rho kinase.[14, 15] RhoA binds to Rho kinase only when it is activated as a GTP-bound form.[5] When activated, RhoA changes its location primarily from cytosol to plasma membrane. Among various substrates, Rho kinase regulates phosphorylation levels of ERM proteins either directly or indirectly by negative regulation of

myosin phosphatase.[5, 16-19] Even with their important role in cell shape determination and relevant signaling pathway, ERM proteins and RhoA signaling have not been studied yet not only in the NAcc but also in association with cocaine in this site. Although cocaine, as a drug of abuse, develops addiction when it is chronically administered, it will be the first step to get the knowledge about the effect of acute cocaine on the regulation of ERM proteins and RhoA signaling, especially when there is nothing known about those molecules in association with this drug. Thus, it was examined in the present study how a single injection of cocaine regulates the phosphorylation levels of ERM proteins and RhoA activity in the NAcc.

Ⅱ. MATERIALS AND METHODS

1. Subjects

Male Sprague-Dawley rats weighing 220-250 g on arrival were obtained from Orient Bio Inc. (Seongnam-si, Korea). They were housed three per cage in a 12-hrs light/dark cycle room (lights out at 8:00 pm) and all experiments were conducted during the day time. Rats had access to water and food *ad libitum* at all times. All animal use procedures were conducted according to an approved Institutional Animal Care and Use Committee protocol.

2. Drugs

Cocaine hydrochloride (Belgopia, Belgium) was dissolved in sterile 0.9% saline. Y27632 dihydrochloride (Tocris Cookson, Bristol, UK) and Rho-Kinase Inhibitor II (RKI II) (Calbiochem, La Jolla, CA, USA) were dissolved in sterile 0.9% saline and 100% DMSO, respectively, and small aliquots were stored at -80℃. Immediately before use, frozen aliquots of each drug were diluted to final working concentrations of 2.0 or 20.0 μg/μl in 0.9% saline (Y27632) and of 1.0 or 4.0 μg/μl in 80% DMSO (RKI II).

3. Brain tissue preparation

For Figure 1B experiments, animals were decapitated at different time points (0, 5, 15, 30, and 60 min) after saline or cocaine IP injections. For all other experiments, animals were decapitated 15 min after either IP or microinjections. Brains were rapidly removed and coronal sections (1.0 mm thick extending 1.60 – 2.60 mm from bregma) were obtained with an ice-cold brain slicer. Tissue punches (1.2 mm diameter) were obtained in the NAcc region (covering most medial part of the core and some portion of the shell near the border of the two) on an ice-cold plate, immediately frozen on dry ice and stored at –80 $^{\circ}$C. They were prepared bilaterally and pooled for each individual animal's protein isolation.

4. Western blotting

Tissues were homogenized in lysis buffer containing 0.32 M sucrose, 2 mM EDTA, 1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, and 1 mM sodium orthovanadate. The concentration of protein was determined by using Pierce BCA protein assay kit (Pierce, Rockford, IL, USA). Samples were then boiled for 10 min and subjected to SDS-polyacrylamide gel electrophoresis. Proteins

were transferred electrophoretically to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), which were then blocked with 5% skim milk in PBS-T buffer [10 mM phosphate-buffered saline plus 0.05% Tween-20]. Specific antibodies against total ERM (1:1,000 dilution in PBS-T with 5% bovine serum albumin; Cell Signaling, Beverly, MA, USA), phospho-ERM (specific to detect phosphorylated ezrin-radixin-moesin at threonine 567, 564 or 558, respectively; 1:500 dilution in PBS-T with 5% bovine serum albumin; Cell Signaling, Beverly, MA, USA), RhoA (1:500 dilution in PBS-T with 5% bovine serum albumin; Cell Signaling, Beverly, MA, USA), phospho-MYPT1 (specific to detect phosphorylated MYPT (myosin-binding subunit of myosin phosphatase) at threonine 696; 1:500 dilution in PBS-T with 5% bovine serum albumin; Upstate Biotechnology, Lake Placid, NY, USA) and anti-β-actin (1:10,000 dilution in PBS-T with 5% skim milk; Abcam, Cambridge, UK) were used to probe the blots. Primary antibodies were detected with peroxidase-conjugated secondary antibodies anti-rabbit IgG (1:2,000 dilution in PBS-T with 5% skim milk; KOMA Biotech, Seoul, Korea) or anti-mouse IgG (1:5,000 dilution in PBS-T with 5% skim milk; Cell Signaling, Beverly, MA, USA) followed by enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, Arlington Heights, IL, USA) and exposure to X-ray film. Band intensities were quantified based on densitometric values using Fujifilm Science Lab 97 Image

Gauge software (version 2.54).

5. RhoA activity assay

RhoA activity was measured by pull-down assay using a fusion protein of glutathione S-transferase (GST) and Rho-binding domain of mouse rhotekin (RBD). Tissues were homogenized in ice-cold Mg^{2+} lysis / wash buffer (MLB) containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5% Triton X-100, 10% glycerol, 1 mM PMSF, 1 mM dithiothreitol, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. Crude homogenates were centrifuged at 10,000 g for 15 min at 4 °C. Supernatants were incubated with GST-RBD coupled glutathione-Sepharose beads (30 μg/sample) for 60 min at 4 °C. Beads were then washed three times with MLB and bound proteins were eluted with Laemmli sample buffer. Samples were separated by SDS-PAGE, and analyzed by immunoblotting with anti-RhoA antibodies.

6. Sub-cellular fractionation of rat brain tissues

We referred to the methods by Dunah and Standaert.[20] Tissues were homogenized in ice-cold TEVP buffer containing 10 mM Tris-HCl (pH 7.4), 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA and 320 mM sucrose. Crude homogenates were centrifuged at 1,000 g for 10 min at 4 °C to remove nuclei and large debris. The supernatant was centrifuged at 10.000 g for 45 min at 4 °C and the resulting supernatant was used as "cytosol" fraction. The pellet was re-suspended in TEVP buffer and centrifuged further at 25,000 g for 45 min at 4 °C and the resulting pellet was used as "membrane" fraction. The fraction samples were then separated by SDS-PAGE and analyzed by immunoblot with anti-RhoA antibodies.

7. Surgical procedures

Rats were anesthetized with intraperitoneal (IP) ketamine (100 mg/kg) and xylazine (6 mg/kg), placed in a stereotaxic instrument with the incisor bar at 5.0 mm above the interaural line and implanted with chronic bilateral guide cannulas (22 gauge; Plastics One, Roanoke, VA, USA) aimed at the core of the NAcc (A/P, +3.4; L, ±1.5; D/V, –7.5 mm from bregma and skull).[21] Cannulas were angled at 10 ° to the vertical, positioned 1 mm above the final injection

site, and secured with dental acrylic cement anchored to stainless steel screws fixed to the skull. After surgery, 28 gauge obturators were placed in the guide cannulas, and rats were returned to their home cages for a 7-day recovery period.

8. Intracranial microinjections

Bilateral intracranial microinjections into the NAcc were made in the freely moving rat. Injection cannulas (28 gauge) connected to 1 µl syringes (Hamilton, Reno, NV, USA) via PE-20 tubing were inserted to a depth 1 mm below the guide cannula tips. Injections were made in a volume of 0.5 µl per side over 30 sec. After 1 min, the injection cannulas were withdrawn and the obturators were replaced.

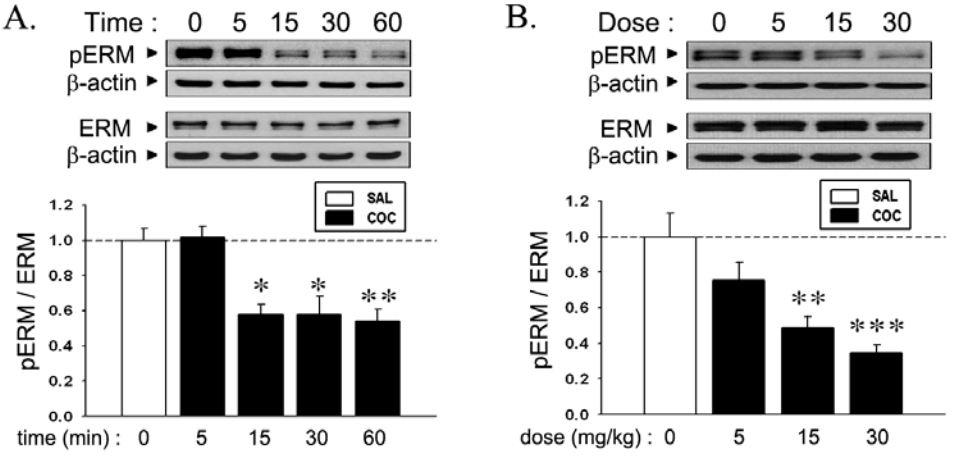
9. Statistical analyses

The data were analyzed with one-way ANOVA (analysis of variance). Post-hoc Scheffé comparisons were made according to Kirk.[22] Differences between experimental conditions were considered statistically significant when $P < 0.05$.

Ⅲ. RESULTS

1. Cocaine decreases the phosphorylation levels of ERM proteins in the NAcc

In order to test whether cocaine may regulate ERM proteins in the NAcc, the ratio of the phosphorylated to total ERM protein levels was examined by immunoblot method with the NAcc tissues obtained at various time points after a single IP injection of cocaine (15 mg/kg). Post-hoc *Scheffé* comparisons after one-way ANOVA [$F_{4, 20} = 11.60$, $p < 0.001$] conducted on these data revealed that the ratio of the phosphorylated to total ERM was significantly reduced ($p <$ 0.05 – 0.01) in cocaine compared to saline administered rats at between 15 and 60 min time points measured (Fig. 1A). Next, the effects of different doses of cocaine (5, 15 or 30 mg/kg) on the levels of ERM proteins was examined with the tissues obtained at 15 min after cocaine injection. As shown in Fig. 1B, a single IP injection of cocaine dose-dependently decreases the ratio of the levels of phosphorylated to total ERM in the NAcc [$F_{3, 24} = 11.40$, $p < 0.001$].


A.
Time : 0 5 15 30 60
pERM ▶
β-actin ▶
ERM ▶
β-actin ▶
SAL
COC
pERM / ERM
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
**
time (min) : 0 5 15 30 60
B.
Dose : 0 5 15 30
pERM ▶
β-actin ▶
ERM ▶
β-actin ▶
SAL
COC
pERM / ERM
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
***
dose (mg/kg) : 0 5 15 30

C.

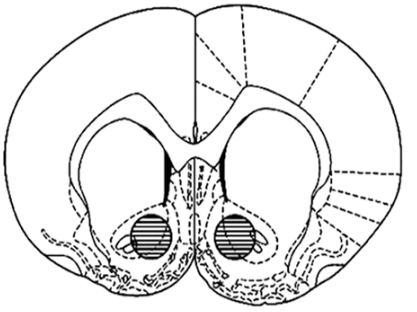


Figure 1. Cocaine decreases the phosphorylation levels of ERM protens in the NAcc. A-B, Representative Western blots were shown. Values for the band intensities normalized to β-actin were expressed as mean ± s.e.m. relative to saline control. Symbols indicate significant differences as revealed by *post-hoc* Scheffé comparisons following one-way ANOVA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; significantly different from saline (n=5 and 7 per group for A and B, respectively). C, The NAcc region where tissues were punched out is shown (cross-hatched circles). Punches (1.2 mm diameter) were prepared bilaterally and pooled for each individual animal's protein isolation. Line drawing is from Paxions and Watson[23] and depicts the caudal surface of a coronal section (1.0 mm thick) extending 1.60 – 2.60 mm from bregma.

2. Cocaine dose-dependently decreases active RhoA levels in the NAcc

Next, active RhoA levels in the NAcc were examined after cocaine injection. When a pull-down assay was performed for endogenous active RhoA in the NAcc tissue obtained 15 min after a single cocaine (15 or 30 mg/kg, IP) injection, the ratio of the GTP bound (active) to the total RhoA was significantly decreased in cocaine- compared to saline-injected rats as revealed by post-hoc *Scheffé* comparisons after one-way ANOVA [$F_{2, 15} = 29.82$; $p < 0.001$] (Fig. 2A). When activated, RhoA changes its location primarily from cytosol to plasma membrane. In order to further confirm the relative sub-cellular distribution of RhoA, their levels in two different compartments for membrane and cytosol, respectively, were measured (Fig. 2B). These data revealed that the membrane to cytosol ratio of RhoA was significantly reduced ($p < 0.05$) in cocaine (30 mg/kg, but not with 15 mg/kg) compared to saline administered rats. These results indicate that cocaine regulates RhoA in the same direction with ERM proteins resulting in inactivation of them in the NAcc.

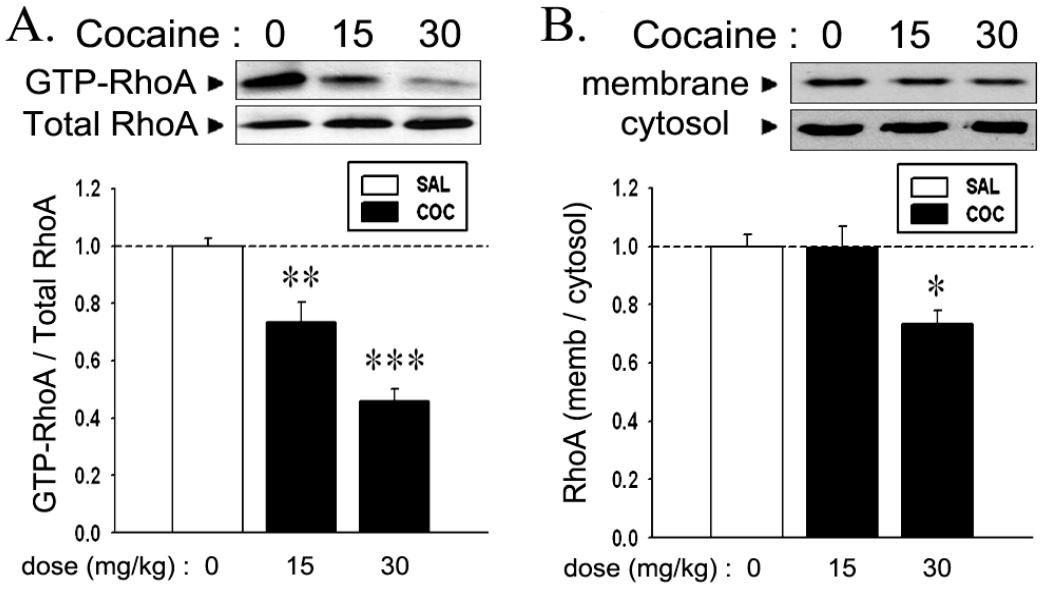


Figure 2. Cocaine dose-dependently decreases active RhoA levels in the NAcc. A-B, Representative Western blots were shown. Values for the band intensities were expressed as mean ± s.e.m. relative to total RhoA (A) or to cytosol fraction (B). Symbols indicate significant differences as revealed by *post-hoc* Scheffé comparisons following one-way ANOVA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; significantly different from saline (n=5 and 6 per group for A and B, respectively).

3. Rho kinase inhibition dose-dependently lowers the phosphorylation levels of ERM proteins in the NAcc

During the course of experiments, it was hypothesized that cocaine-induced inactivation of RhoA and consequently accompanied down-regulation of Rho kinase activity may produce the decrease of phosphorylation levels of ERM proteins. To test this, a bilateral microinjection was made into the NAcc with a specific Rho kinase inhibitor, Y27632 (1.0 or 10.0 μg/0.5 μl/side), and phosphorylated ERM levels were measured in this site. Interestingly, it was found that Y27632 dose-dependently decreased the ratio of phosphorylated to total ERM levels compared to vehicle in the NAcc as revealed by post-hoc *Scheffé* comparisons after one-way ANOVA [$F_{2, 24} = 9.55$; $p < 0.001$] (Fig. 3A). To avoid a possible interpretation that the effects of Y27632 may be due to its inhibitory property for other protein kinases (e.g., protein kinase C-related kinase),[5, 24] a separate microinjection experiment was conducted with another Rho kinase inhibitor, RKI II, known to be highly specific to this enzyme.[19] It was also found that RKI II (0.5 or 2.0 μg/0.5 μl/side) decreased the ratio of phosphorylated to total ERM levels in the NAcc [$F_{2, 9} = 7.68$; $p < 0.05$] (Fig. 3B), which confirms that the specific inhibition of Rho kinase in the NAcc has a negative regulatory effect on ERM phosphorylation levels in this site. These

results suggest that cocaine-induced decrease of phosphorylated ERM levels may be produced by down-regulation of Rho kinase activity following RhoA inactivation.

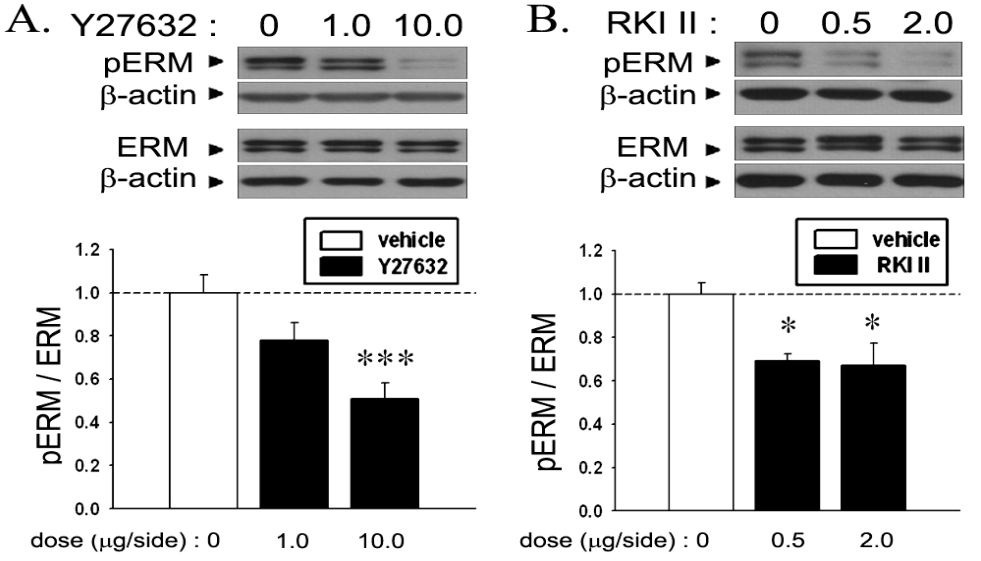


Figure 3. Microinjection of Rho kinase inhibitors into the NAcc lowers phosphorylation levels of ERM in this site. A-B, Representative Western blots were shown. Values for the band intensities normalized to β-actin were expressed as mean ± s.e.m. relative to vehicle control. Symbols indicate significant differences as revealed by *post-hoc* Scheffé comparisons following one-way ANOVA. * $p<0.05$, *** $p<0.001$; significantly different from vehicle (n=9 and 4 per group for A and B, respectively).

4. Myosin phosphatase may not be involved in cocaine-induced decrease of ERM phosphorylation levels in the NAcc

It is possible that cocaine-induced decrease of ERM phosphorylation levels in the NAcc may due to up-regulation of myosin phosphatase activity, which is under negative regulation of Rho kinase.[5, 16] In order to verify whether this is the case, phosphorylated MYPT (myosin-binding subunit of myosin phosphatase) levels were measured in the NAcc after Y27632 microinjection (1.0 and 10.0 μg/0.5 μl/side) or cocaine (15 or 30 mg/kg, IP) injection. As expected, Y27632 dose-dependently decreased phosphorylated MYPT levels compared to saline as revealed by post-hoc *Scheffé* comparisons after one-way ANOVA [$F_{2, 24} = 5.91$; $p < 0.008$] (Fig. 4A). Interestingly, however, cocaine produces no change of phosphorylated MYPT levels in this site (Fig. 4B), suggesting that cocaine-induced regulation of phosphorylated ERM levels in the NAcc may not be relevant with myosin phosphatase and rather be more likely under direct effect of Rho kinase activity as shown in Fig. 3.

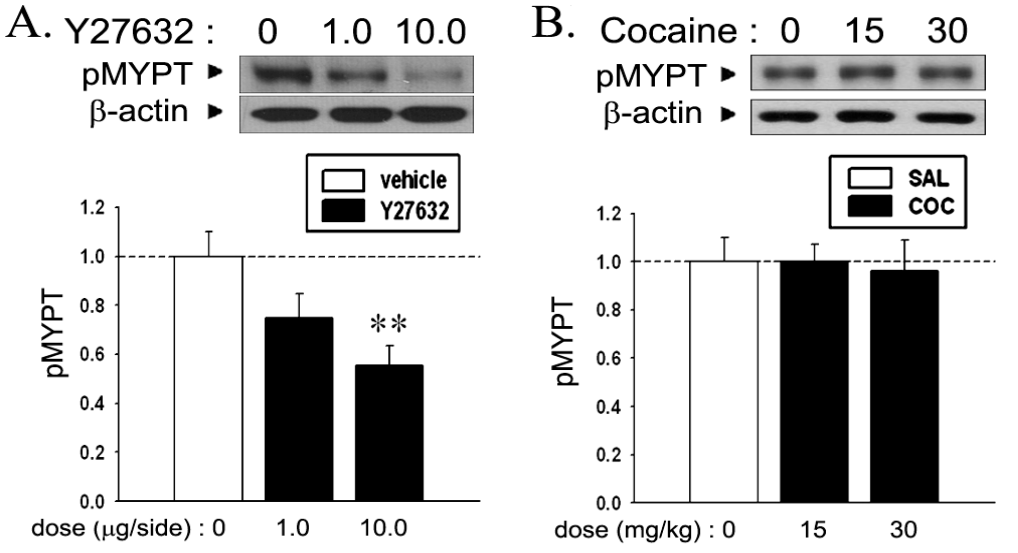


Figure 4. The phosphorylation levels of MYPT in the NAcc are differentially regulated by Rho kinase inhibitor and cocaine. A-B, Representative Western blots were shown. Values for the band intensities normalized to β-actin was expressed as mean ± s.e.m. relative to either vehicle (A) or saline (B) control. Symbols indicate significant differences as revealed by *post-hoc* Scheffé comparisons following one-way ANOVA. ** $p<0.01$; significantly different from vehicle microinjection group. (n=9 per group for both A and B).

IV. DISCUSSION

In the present experiments, it was shown first time, to my knowledge, that cocaine, even a single injection, can regulate the cellular status of structurally important ERM proteins by influencing RhoA-Rho kinase signaling in the NAcc. Considering the role of ERM proteins in the cell-shape determination by crosslinking F-actin to plasma membrane,[9, 10] these results suggest that acute cocaine possibly begins cellular events leading to actin cytoskeletal remodeling by regulation of RhoA-ERM signaling in the NAcc (as shown in Fig. 5 and 6). Here I further propose that, when repeatedly exposed to cocaine, this process can be amplified to produce morphologically detectable figures of the structural plasticity which have been shown in animal models of drug addiction such as behavioral sensitization and self-administration.[3, 4, 6]

ERM proteins contain a filamentous F-actin binding site at C-terminal domain and the phosphorylation of the regulatory threonine site in this domain stably maintains F-actin binding and consequently its linkage to the plasma membrane.[9] Although it has not directly measured here, current findings that cocaine reduces ERM stability by decreasing its phosphorylation levels may subsequently result in disruption of ERM and F-actin linkage.

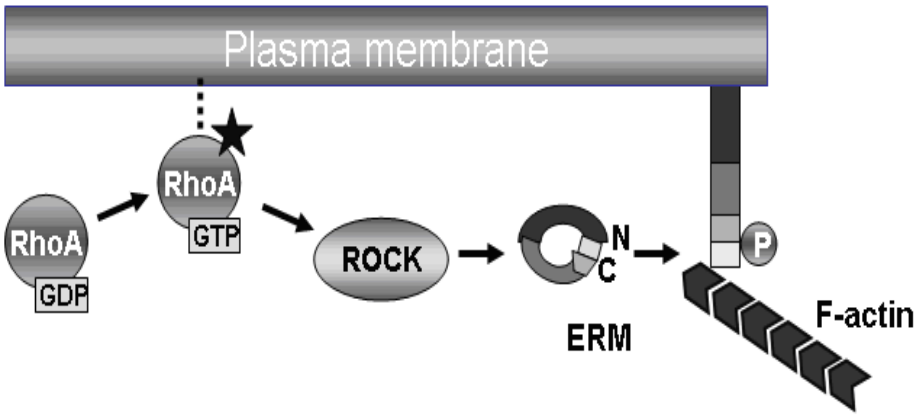


Figure 5. Model describing the RhoA-ROCK signaling pathway leading to activation of ERM protein and its F-actin linkage.

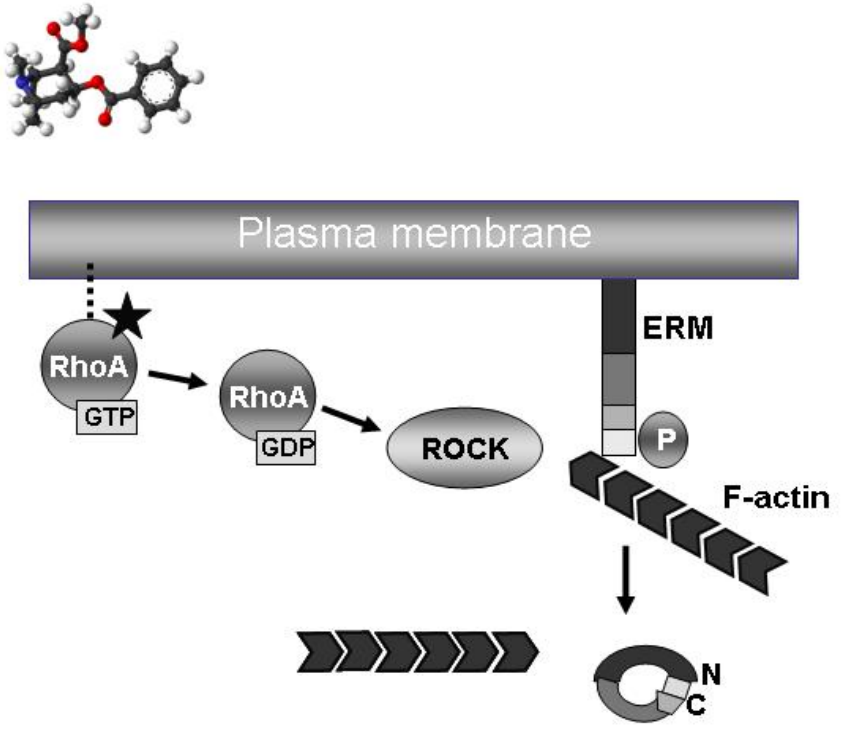


Figure 6. Model describing the RhoA-related signaling pathway leading to disruption of ERM and F-actin linkage. Cocaine seems to affect this pathway in the NAcc when acutely injected.

Interestingly, the amount of F-actin has been previously shown to increase in the NAcc when measured 45 min after acute cocaine (30 mg/kg, IP).[13] Decrease of phosphorylation levels of ERM, as shown in the current findings, and consequently following possible loss of its stability to F-actin linkage may explain why the amount of F-actin increases after acute cocaine within the similar time range (15 to 60 min for ERM and 45 min for F-actin).

It has shown that RhoA and its down-stream effector Rho kinase are primarily responsible for the phosphorylation of the C-terminal domain of ERM proteins.[5, 17-19] In my own experiments, it was shown that microinjection into the NAcc of Rho kinase inhibitors dose-dependently lowers ERM phosphorylation levels in this site (Fig. 3A, B). Further, it was also shown that cocaine dose-dependently decreases active RhoA levels in the NAcc by using GST-RBD pull-down assay (Fig. 2B). Additionally, a differential distribution of active RhoA was detected in membrane compared to cytosol fractions by cocaine (30 mg/kg), although it was not detected by a lower dose of cocaine (15 mg/kg) (Fig. 2A). The different result for lower dose of cocaine may be due to different sensitivity of methods used in this experiment. Together, these results indicate that cocaine decreases active RhoA levels and subsequently Rho kinases in the NAcc in the same direction with the reduction of ERM phosphorylation levels.

There is rich evidence that actin dynamics in dendritic spines are highly associated with spine morphogenesis.[25-27] However, the exact role of RhoA signaling in dendritic spine remodeling and neurite development has been shown to be controversial, either in a positive or negative way, depending on the cell type and context. For example, inhibition of RhoA activity increases dendritic number in cultured hippocampal neurons[28] and increases neuritic development in cultured retina neurons,[29] while a local reduction of RhoA activity results in spine collapse in another hippocampal culture.[30] Interestingly, actin dynamics regulating postsynaptic organization has also been shown to be bidirectional in its plasticity.[31] Thus, because actin dynamics wasn't directly measured here in the present study, it remains to be explored yet to which direction the present findings that cocaine decreases RhoA signaling may lead actin dynamics in terms of dendritic spine remodeling.

It is possible that ERM phosphorylation levels in the NAcc can also be negatively regulated by myosin phosphatase, of which activity is under negative regulation by Rho kinase as well.[5, 16] It was shown, first time here, that microinjection in the NAcc of a Rho kinase inhibitor, Y27632, dose-dependently decreases phosphorylated MYPT levels in this site (Fig. 4A). Interestingly, however, these effects are not present with cocaine injection (Fig. 4B), suggesting that cocaine-induced reduction of phosphorylated ERM levels

in the NAcc may not be indirectly regulated via myosin phosphatase and rather be under more likely direct effect of Rho kinase activity as shown in Fig. 3A, B. However, it needs more experiments to draw more concrete conclusion.

As a dopamine re-uptake blocker, cocaine increases synaptic dopamine levels.[32] However, there is very little information available in the literature that directly shows the relationship between dopamine and RhoA signaling. Interestingly, one study showed that inactivation of RhoA induced a morphological change similar to that produced by dopamine in anterior pituitary lactotropes.[33] In the present study, dopamine would possibly be increased by cocaine and it may directly or indirectly influence cell structural remodeling in the same direction with RhoA. However, these are very much speculative and it remains to be investigated in the future whether there might be an ongoing interaction between dopamine and RhoA signaling pathways.

In the present experiments, protein analysis was performed with the NAcc tissues punched out including mostly core and some portion of shell sub-regions. However, considering the expanding knowledge for the differential involvement in addiction-related behaviors of the two sub-regions, core and shell, in the NAcc,[34, 35] it will be needed a more thorough systematic assessment of the effects of cocaine on the regulation of ERM and RhoA signaling in these different sub-regions of the NAcc in near future.

Although the details remain to be investigated, the present findings suggest that ERM proteins, one of the important controllers of the cytoskeleton, may reside in a critical position mediating cocaine-induced structural plasticity, possibly via inactivation of RhoA-Rho kinase signaling. In relevance with my study, there have been some interesting studies showing that RhoA gene is associated with smoking initiation by microarray analysis of acute nicotine responses in mouse brain,[36] while ethanol impairs Rho GTPase signaling in a rodent model of fetal alcohol syndrome.[37] Thus, it will be interesting to examine in the future whether other types of drugs of abuse also produce similar findings in the NAcc as proposed here.

V. CONCLUSION

The present results suggest that even acute injection of cocaine is able to initiate possible cellular events leading to actin cytoskeletal remodeling by regulation of RhoA-ERM signaling in the NAcc. Interestingly, it has recently shown that the NAcc dendritic spine density is actually increased 6 hours after even a single cocaine injection.[7] It is plausible that such a transient change becomes a form of metaplasticity, i.e., the alterations in biochemical or morphological processes that change the synaptic ability to generate more endurable plasticity,[8] when we consider, for example, the fact that the increase of dopamine levels by acute cocaine develops later to the sensitized increase in response to the same dose of cocaine when it is repeatedly administered.[31, 38] This process may possibly be mediated by a sequence of biochemical changes.[1, 39] Thus, here I propose that acute cocaine can induce a form of metaplasticity that alters synaptic ability to respond later in amplification, when animals are repeatedly exposed to cocaine, resulting in some morphologically better detectable figures as shown in animal models of drug addiction such as behavioral sensitization and self-administration.[3, 4, 6, 40]

REFERENCES

1. Koob GF, Le Moal M. Drug addiction, dysregulation of reward and allostasis. Neuropsychopharmacology 2001;24:97-129.

2. Kalivas PW, Volkow ND. The neural basis of addiction: a phathology of motivation and choice. Am J Psychiatry 2005;162:1403-13.

3. Robinson, TE, Gorny G, Mitton E, Kolb B. Cocaine self-administration alters the morphology of dendrites and dendritic spines in the nucleus accumbens and neocortex. Synapse 2001;39:257-66.

4. Li Y, Acerbo MJ, Robinson TE. The induction of behavioral sensitization is associated with cocaine-induced structural plasticity in the core (but not shell) of the nucleus accumbens. Eur J Neurosci 2004;20:1647-54.

5. Riento K, Ridley AJ. ROCKs: Multifunctional kinases in cell behaviour. Nature Rev Mol Cell Biol 2003;4:446-56.

6. Lee K-W, Kim Y, Kim AM, Helmin K, Nairn AC, Greengard P. Cocaine-induced dendritic spine formation in D1 and D2 dopaminergic receptor-containing medium spiny neurons in nucleus accumbens. Proc Natl Acad Sci USA. 2006;103:3399-404.

7. Shen H, Toda S, Moussawi K, Bouknight A, Zahm DS, Kalivas PW. Altered dendritic spine plasticity in cocaine-withdrawn rats. J Neurosci 2009;29:2876-884.

8. Abraham WC. Metaplasticity: tuning synapses and networks for plasticity. Nature Rev Neurosci 2008;9:387-99.

9. Bretscher A, Edwards K, Fehon RG. ERM proteins and merlin: Integrators at the cell cortex. Nature Rev Mol Cell Biol 2003;3:586-99.

10. Niggli V, Rossy J. Ezrin/radixin/moesin: Versatile controllers of signaling molecules and of the cortical cytoskeleton. Int J Biochem Cell Biol 2008;40:344-49.

11. Paglini G, Beeser A, Chernoff J, Testa JR. Suppression of radixin and moesin alters growth cone morphology, motility, and process formation in primary cultured neurons. J Cell Biol 1998;143:443-55

12. Matus A. Growth of dendritic spines: a continuing story. Curr Opin Neurobiol 2005;15:67-72.

13. Toda S, Shen H, Peters J, Cagle S, Kalivas PW. Cocaine increases actin cycling: Effects in the reinstatement model of drug seeking. J Neurosci 2006;26:1579-87.

14. Hall A. Rho GTPases and the actin cytoskeleton. Science 1998;279:509-14.

15. Brown JH, Del Re DP, Sussman MA. The Rac and Rho hall of fame. Circ Res 2006;98:730-42.

16. Fukata Y, Kimura K, Oshiro N, Saya H, Matsuura Y, Kaibuchi K. Association of the myosin-binding subunit of myosin phosphatase and moesin: Dual regulation of moesin phosphorylation by Rho-associated kinase and myosin phosphatase. J Cell Biol 1998;141:409-18.

17. Jeon S, Kim S, Park J-B, Suh P-G, Kim YS, Bae C-D et al. RhoA and Rho

kinase-dependent phosphorylation of moesin at Thr-558 in hippocampal neuronal cells by glutamate. J Biol Chem 2002; 277:16576-84.

18. Yonemura S, Matsui T, Tsukita S, Tsukita S. Rho-dependent and – independent activation mechanisms of ezrin/radixin/moesin proteins: an essential role for polyphosphoinositides in vivo. J Cell Sci 2002;115:2569-80.

19. Haas MA, Vickers JC, Dickson TC. Rho kinase activates ezrin/radixin/moesin proteins and mediates their function in cortical neuron growth, morphology and motility in vitro. J Neurosci Res 2007;85:34-46.

20. Dunah AW, Standaert DG. Dopamine D1 receptor-dependent trafficking of striatal NMDA glutamate receptors to the postsynaptic membrane. J Neurosci 2001;21:5546-58.

21. Pellegrrino LJ, Pellegrino AS, Cushman AJ. A stereotaxic atlas of the rat brain. New York: Plenum; 1979.

22. Kirk RE. Experimental design: procedures for the behavioral sciences. Belmont, CA: Brooks/Cole; 1968.

23. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. Amsterdam: Elsevier Academic; 1977.

24. Darenfed H, Dayanandan B, Zhang T, Hsieh SHK, Fournier AE, Mandato CA. Molecular characterization of the effects of Y-27632. Cell Motil Cytoskel 2007;64:97-109.

25. Matus A, Brinkhaus H, Wagner U. Actin dynamics in dendritic spines: A form of regulated plasticity at excitatory synapses. Hippocampus

2000;10:555-60.

26. Sekino Y, Kojima N, Shirao T. Role of actin cytoskeleton in dendritic spine morphogenesis. Neurochem Int 2007;51:92-104.

27. Zito K, Knott G, Shepherd GMG, Shenolikar S, Svoboda K. Induction of spine growth and synapse formation by regulation of the spine actin cytoskeleton. Neuron 2004;44:321-34.

28. Chen H, Firestein BL. RhoA regulates dendritic branching in hippocampal neurons by decreasing cypin protein levels. J Neurosci 2007;27:8378-86.

29. Fontainhas AM, Townes-Anderson E. RhoA and its role in synaptic structural plasticity of isolated salamander photoreceptors. Invest Ophthalmol Vis Sci 2008;49:4177-87.

30. Schubert V, Da Silva JS, Dotti CG. Localized recruitment and activation of RhoA underlies dendritic spine morphology in a glutamate receptor-dependent manner. J Cell Biol 2006;172:453-67.

31. Okamoto K-I, Nagai T, Miyawaki A, Hayashi Y. Rapid and persistent modulation of actin dynamics regulates postsynaptic reorganization underlying bidirectional plasticity. Nature Neurosci 2004;7:1104-12.

32. Hurd YL, Weiss F, Koob GF, Anden N-E, Ungerstedt U. Cocaine reinforcement and extracellular dopamine overflow in the rat nucleus accumbens: an in vivo microdialysis study. Brain Res 1989;498:199-203.

33. Nguyen B, Carbajal ME, Vitale ML. Intracellular mechanisms involved in dopamine-induced actin cytoskeleton organization and maintenance of a round

phenotype in cultured rat lactotrope cells. Endocrinology 1999;140:3467-77.

34. Di Chiara G. Nucleus accumbens shell and core dopamine: Differential role in behavior and addiction. Behav Brain Res 2002;137:75-114.

35. Zahm D. An integrative neuroanatomical perspective on some subcortical substrates of adaptive responding with emphasis on the nucleus accumbens. Neurosci Biobehav Rev 2002;24:85-105.

36. Chen X, Che Y, Zhang L, Putman AH, Damaj I, Martin BR et al. RhoA, encoding a Rho GTPase, is associated with smoking initiation. Genes Brain Behav 2006;6:689-97.

37. Joshi S, Guleria RS, Pan J, Bayless KJ, Davis GE, DiPette D et al. Ethanol impairs Rho GTPase signaling and differentiation of cerebellar granule neurons in a rodent model of fetal alcohol syndrome. Cell Mol Life Sci 2006;63:2859-70.

38. Kalivas PW, Stewart J. Dopamine transmission in the initiation and expression of drug and stress-induced sensitization of motor activity. Brain Res Rev 1991;16:223-44.

39. Nestler EJ. Is there a common molecular pathway for addiction? Nature Neurosci 2005;8:1445-49.

40. Robinson TE, Kolb B. Structural plasticity associated with exposure to drugs of abuse. Neuropharmacology 2004;47:33-46.

Abstract (in Korean)

중격측좌핵에서 코카인에 의한 ezrin-radixin-moesin 과 RhoA의 신호전달 조절기전에 대한 연구

지도교수 김 정 훈

연세대학교 대학원 의과학과

김 화 영

코카인은 중뇌의 복측피개영역에서 전뇌의 중격측좌핵으로 연결되는 보상회로라고 불리는 신경회로망에 영향을 미치는데, 이중에서도 중격측좌핵은 복측피개영역 뿐 아니라 전전두엽 피질 등 여러 뇌 부위에서 정보를 받아 코카인에 의한 행동변화를 나타내는 부위이다. 실험동물에게 코카인을 반복하여 주면 중격측좌핵 신경세포에 구조 및 기능에 변화가 생기면서 중독이라는 병적 상태에 빠지게 된다. 실제로 행동과민반응과 같은 약물중독 동물 모델에서 수상돌기 가시의 수가 많아지는 것이 중격측좌핵에서 관찰되었음이 보고 된 바 있다. 수상돌기 가시는 축삭종말과 시냅스를 이루며, 액틴미세섬유가 많이 존재한다고 알려져 있다. Ezrin-radixin-moesin (ERM) 단백질은 서로 높은 상동성을 가지고 있으며, 구조 단백질인 섬유성 액틴 단백질을 원형질 막과 연결 시켜주는 역할을 하면서 수상돌기 가시의 형태를 조절하는데 관여한다. 이러한 지식을 배경으로 본 연구에서는 중격측좌핵에서 ERM 단백질의 인산화가 코카인에 의해 어떻게 조절을 받는가에 대해 알아보았다.

우선, 코카인을 복강주입 하였을 때, 주입 후 15분부터 60분까지 코카인 농도 의존적으로 중격측좌핵의 ERM 단백질의 인산화가

현저하게 감소함을 관찰하였다. ERM 단백질은 C 말단의 스레오닌(threonine)이 인산화 되어야 활성화 되는데, 이 부위의 인산화는 RhoA와 이것의 가장 유력한 기질인 Rho kinase에 의하여 조절 받는다는 것이 알려져 있다. RhoA는 GTP와 붙어 있을 때만 활성화 되며, 동시에 Rho kinase와 결합한다. 또한 이렇게 활성화 된 RhoA와 Rho kinase는 세포막으로 이동하게 된다. 이러한 배경에 따라 다음으로 원형질막으로 이동한 RhoA양과 GTP와 결합한 RhoA양을 조사하였다. 그 결과, ERM 인산화와 마찬가지로 원형질막으로 이동한 RhoA 뿐 아니라 GTP와 결합한 RhoA 양이 코카인에 의해 감소함을 관찰하였다. 또한, Rho kinase의 억제제 Y27632 (1.0 or 10.0 μg/0.5 μl/side) 또는 RKI II (0.5 or 2.0 μg/0.5 μl/side)를 직집 중격측좌핵에 주입하였더니, 역시 ERM의 인산화가 감소함을 관찰하였다.

이러한 결과들을 종합해보면, 중독성 약물인 코카인은 중격측좌핵의 ERM과 RhoA, 그리고 Rho kinase에 의한 신호전달경로를 조절하고 있음을 알 수 있다. ERM 단백질은 세포 내에서 신호전달에 관여할 뿐 아니라, 세포 구조의 유지에도 중요한 역할을 담당하고 있기 때문에 이러한 결과들은 중독성 약물이 어떤 기전으로 중격측좌핵 신경세포의 구조변화를 일으키는가에 대한 중요한 단서를 제공할 수 있을 것으로 사료된다.

핵심 되는 말: 코카인, ERM, RhoA, 중격측좌핵, 구조적 변화, 약물 중독

Publication list

1. **Wha Young Kim**, Seungwoo Kim, Jeong-Hoon Kim. Chronic microinjection of valproic acid into the nucleus accumbens attenuates amphetamine-induced locomotor activity. *Neurosci Lett* 2008;432:54-7.

2. **Wha Young Kim**, Paul Vezina, Jeong-Hoon Kim. Blockade of group II, but not group I, mGluRs in the rat nucleus accumbens inhibits the expression of conditioned hyperactivity in an amphetamine-associated environment. *Behav Brain Res* 2008;191:62-6.

3. **Wha Young Kim**, So Ra Shin, Seungwoo Kim, Songhee Jeon, Jeong-Hoon Kim. Cocaine reduces the phosphorylation levels of ezrin-radixin-moesin proteins and RhoA activity in the rat nucleus accumbens. *Neuroscience* 2009;163:501-5.

4. Hyung Shin Yoon, **Wha Young Kim**, Jeong-Hoon Kim. Microinjection of CART peptide 55-102 into nucleus accumbens inhibits the expression of conditioned hyperactivity in a cocaine-associated environment. *Behav Brain Res* 2010;207:169-173.