Extracellular signal-regulated kinase signaling in the nucleus accumbens and behavioral sensitization by cocaine

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Extracellular signal-regulated kinase signaling in the nucleus accumbens and behavioral sensitization by cocaine

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

Seungwoo Kim

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ABSTRACT

Extracellular signal-regulated kinase signaling in the nucleus accumbens and behavioral sensitization by cocaine

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Repeated administration of cocaine leads to the development of behavioral sensitization. Mitogen–activated protein (MAP) kinases play a crucial role in cell growth, proliferation and neuronal plasticity. Extracellular signal-regulated kinase (ERK), one of the subtypes in MAP Kinases, has been implicated in several neurobiological processes. Although the nucleus accumbens (NAcc) is the site mediating the expression of behavioral sensitization by drug of abuse, the precise role of ERK activation in this site has not been determined. In this study we demonstrated that blockade of ERK phosphorylation in the NAcc by a single bilateral microinjection of PD98059 (0.5 or 2.0 μg/side) or U0126 (0.1 or 1.0 μg/side), into this site dose-dependently inhibited the expression of cocaine-induced behavioral sensitization when measured at day 7 following 6 consecutive daily cocaine injections (15mg/kg, IP). This effect appears same when measured after 3 weeks of withdrawal. Acute microinjection of either vehicle or PD98059 alone produced no different locomotor activity compared to saline control. Microinjection of PD98059 (2.0 μg/side) in the NAcc specifically reduced cocaine-induced increase of ERK phosphorylation level in this site, while unaffecting p38 protein levels. Further, we sought to determine

whether ERK activation in the NAcc showed time-dependent changes after cocaine withdrawals. The basal levels of ERK phosphorylation in the NAcc showed no changes on withdrawal day 1, while they increase on day 7, then gradually lower down to reach the same level on day 21 in cocaine compared to saline pre-exposed rats. Either total ERK or both phosphorylated and total p38 protein level were not different in any time-point measurements. Taken together, our results suggest that ERK activation in the NAcc is necessary for the expression of cocaine-induced behavioral sensitization and time-dependent ERK activation in the NAcc may contribute to neuronal plasticity leading to long-lasting behavioral changes such as drug craving.

Key words: cocaine, ERK, behavioral sensitization, nucleus accumbens, microinjection, craving

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Ⅰ. INTRODUCTION

Drug abuse is defined as a chronic, relapsing brain disease that is characterized by compulsive drug seeking and use.[1] It is known to be mediated by rewarding circuit in the several brain regions including ventral tegmental area (VTA), nucleus accumbens (NAcc), and prefrontal cortex (PFC).[2] Drug abuse is considered as a brain disease resulting in the long lasting changes of not only brain structures but also molecular and cellular mechanisms.[2] These changes lead eventually to the complex behaviors (for example, dependence, tolerance, sensitization, and craving) that characterize an addicted state. Psychomotor stimulant drug induced-behavioral sensitization is a well-established phenomenon.[3] Repeated intermittent administration of psychomotor stimulant drugs such as cocaine or amphetamine produces behavioral sensitization, which is a proposed model for understanding drug abuse. Behavioral sensitization has been proposed to underlie the ability of psychomotor stimulants to elicit craving by enhancing the incentive motivational value of these drugs.[4] Thus, elucidating the molecular mechanisms

that involve in making these processes provides help to better understanding about the critical steps that mark the transitional shift to compulsive drug use among human addicts.

The extracellular signal-regulated kinase (ERK), a downstream substrate of a mitogen-activated protein kinase kinase (MEK), is a protein serine/threonine kinase that plays an important role in diverse neuronal adaptive responses such as learning and memory.[5, 6] Several lines of evidence indicate that ERK pathway also importantly involves in both acute and long-term adaptive processes by drugs of abuse. For example, the systemic injection of a selective inhibitor of MEK abolishes the rewarding effects as well as the sensitized locomotor activity of cocaine.[7-10] Chronic administration of cocaine or morphine increases ERK activity in the VTA, the region in the ventral midbrain that contains dopaminergic cell bodies.[11] In the NAcc, which receives dopaminergic axonal projections from the VTA, ERK activation is induced by cocaine[8] and it is augmented in cocaine-sensitized rats.[12] Behavioral studies also show that the systemic injection of SL327, a selective inhibitor of MEK, abolished the development of locomotor sensitization as well se conditioned place preference by cocaine.[8, 9]

The development of behavioral sensitization by psychomotor stimulant drugs consists of two phases; induction and expression, in which distinct neuronal substrates mediate different neuronal processes in the brain.[13, 14] Evidence indicates that the development of behavioral sensitization is initiated in the VTA, while its expression is due to drug actions in the NAcc. [5, 13] Recent research shows that the development of sensitization to cocaine was prevented by pre-treatment of a MEK inhibitor prior to each drug administration during the induction phase, [9, 15] while its expression was not affected when challenged later with cocaine in the presence of the same inhibitor.[9] However, these results were all obtained with systemic injection of a MEK inhibitor SL327, which crosses the blood-brain barrier and affects various brain regions, so that it was not clear in which area of brain ERK actually contributes to sensitization. Although it

was previously observed that the initiation of behavioral sensitization to cocaine was blocked by intra-VTA microinjection of another MEK inhibitor PD98059,[7] the role of ERK in the expression of sensitization has not been examined yet directly in the NAcc. Thus, we investigated in the present study whether a specific blockade of ERK phosphorylation by microinjection of PD98059 into the NAcc produces a differential effect to the expression of behavioral sensitization to cocaine.

Interestingly, it has been suggested in both human addicts and laboratory animals that cocaine craving progressively develops or incubates during drug-free withdrawal periods. During drug-free withdrawal periods, what molecular changes contribute to the incubation of craving and subsequently the expression of sensitization is not precisely known yet. Recent research shows that the cell surface expression of AMPA receptors in the NAcc increases during 7–21 days, but not 1 day, of cocaine withdrawals.[16, 17] These results suggest that the drug-free withdrawal periods are not dormant but rather actively changing some molecular expression levels to contribute to the incubation of craving.

Ⅱ. MATERIALS AND METHODS

1. Drugs and intracranial microinjections

PD98059 and U0126 (Sigma, St. Louis, MO) were dissolved in 70% and 20% DMSO, respectively, and small aliquots were stored at -20℃. Immediately before use, frozen aliquots of each drug were diluted to concentrations of either 1.0 or 4.0 μg/μl in 70% DMSO (PD98059) and of either 0.2 or 2.0 μg/μl in 20% DMSO (U0126). Both U0126 and PD98059 are noncompetitive inhibitors with respect to both MEK substrates, ATP and ERK. Cocaine hydrochloride (Belgopia, Belgium) was dissolved in sterile 0.9% saline. Bilateral intracranial microinjections into the NAcc were made in the freely moving rat. Injection cannulas (28gauge) connected 1 μl syringes (Hamilton, Reno, NV) via PE-20 tubing were inserted to a depth 1mm 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.

2. Subject and surgery

Male Sprague–Dawley rats weighing 220–260 g on arrival were obtained from Samtako (Osan, Korea). They were housed three per cage in a 12-h light: 12-h dark cycle room with food and water available at all times. They were allowed to stay in the colony room at least for a week, during which they were handled, before doing any experiments. All animal use procedures were conducted according to an approved IACUC protocol.

During the surgical operation, rats were anesthetized with ketamine (100mg/kg, i.p.) followed by xylazine (6mg/kg, i.p.), 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) aimed at the NAcc (A/P, +3.4; L, ±1.5; D/V, −7.5 mm from bregma and skull). Cannulas were angled at 10° to the vertical, positioned 1mm 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 returned to their home cages allowing full recovery until a challenge test day which lasted at least two weeks.

3. Locomotor activity

Locomotor activity was measured in a bank of six activity boxes (35 cm × 25 cm × 40 cm) (IWOO Scientific Corporation, Seoul, Korea) made of translucent Plexiglas and individually kept in larger PVC plastic sound attenuating cubicles. The floor of each box consisted of 21 stainless steel rods (5 mm diameter) spaced 1.2 cm apart center-to-center. Two infrared light photocells (Med Associates, St. Albans, VT, USA) positioned 4.5 cm above the floor and spaced evenly along the longitudinal axis of the box estimated horizontal locomotor activity.

4. Western blotting

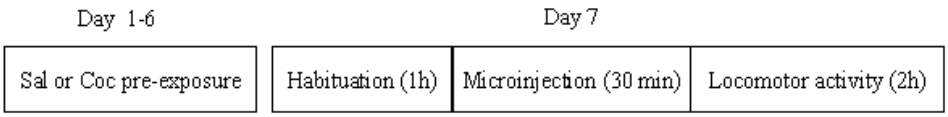
Tissues were homogenized in lysis buffer containing 0.32 M sucrose, 2 mM EDTA, 1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The concentration of protein was determined by using Pierce BCA protein assay kit (Pierce, Rockford, IL, USA). Samples were then boiled 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 phospho-ERK1/2, phospho-p38 (1:1000 dilution in PBS-T with 5% bovine serum albumin; Cell Signaling, Beverly, MA, USA) and ERK1/2, p38 (1:1000 dilution in PBS-T with 5% skim milk; Cell Signaling) MAP kinases were used to probe the blots. Primary antibodies were detected with peroxidase-conjugated secondary antibodies (1:2000 dilution in PBS-T with 5% skim milk; KOMA Biotech, Seoul, Korea) 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). Antibodies on the membrane were removed by using Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL, USA) and re-probed with anti-β-actin antibody (1:10,000 dilution in PBS-T with 5% skim milk; Abcam, Cambridge, UK).

5. Design and procedures

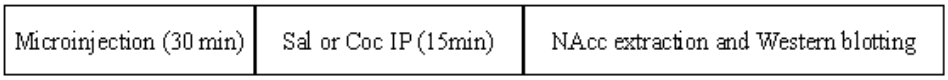
Experiment 1

Six different groups of rats were randomly assigned and half of them were administered once a day with saline and the rest half with cocaine (15mg/kg, IP) for 6 consecutive days. Injections were made in the activity boxes on day 1 and 6, and at home-cage for the rest of days (day 2 to 5), to avoid any confounding factor such as conditioning. On day 7, animals were first habituated to the activity boxes for 1 hour, then microinjected bilaterally into the NAcc with either vehicle (70% DMSO) or each dose of PD 98059 (0.5 and 2.0μg/0.5μl/side) (pre-exposure-microinjection-IP injection; sal-veh-coc, n=6, sal-PD (0.5)-coc, n=6, sal-PD (2.0)-coc, n=6, coc-veh-coc, n=7, coc-PD (0.5)-coc, n=6, coc-PD (2.0)-coc, n=7) and U0126 (0.1 and 1.0 μg/0.5μl/side) (sal-veh-coc, n=5, sal-U0126 (0.1)-coc, n=5, sal-U0126 (1.0)-coc, n=4, coc-veh-coc, n=5, coc-U0126(0.1)-coc, n=6, coc-U0126(1.0)-coc, n=7). After 30 min, they were all cocaine (15mg/kg, IP) challenged and their locomotor activity measured for 2 hours. Additional four groups of rats were first habituated to the activity boxes for 1 hour, then microinjected with either saline, vehicle, or one of two doses of PD98059 (0.5 and 2.0μg/side), followed by saline (IP) challenges 30min later and their locomotor activity measured for 2 hours (n=5 each).



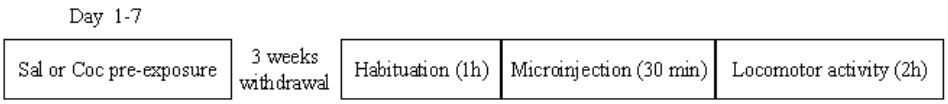
Experiment 2

Two groups of rats were microinjected bilaterally into the NAcc with either vehicle (70% DMSO) or PD 98059 (2.0μg/side), respectively. Then, each group was subdivided into two, which received IP saline or cocaine 30min later (microinjection-IP injection; veh-sal, n=7, veh-coc, n=8, PD-sal, n=7, PD-coc, n=8). Brain tissues dissected immediately after decapitation at 15min after the IP injections, and processed for western blotting.



Experiment 3

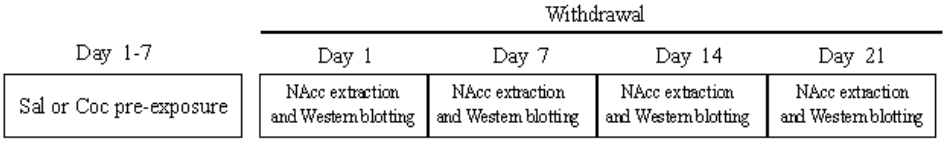
In the long-term withdrawal experiments, three groups of rats (n=4 each) were all cocaine pre-exposed, then after 3weeks of withdrawal, they were either saline or cocaine challenged 30min after microinjection of saline or a high dose of PD98059 (2.0μg/side) and their locomotor activity measured for 2 hours.



Experiment 4

Rats (n=48) were administered with either saline or cocaine (15 mg/kg, i.p.) once daily for 7 days. On days 1 and 7, they were first habituated to the activity boxes for an hour, then injections made and immediately their locomotor activities were measured for an hour, while on day 2 through 6, they were

injected in home cages without activity measurements. After pre-exposures, animals were allowed to have 1, 7, 14 or 21 days of drug-free withdrawal periods. On each scheduled day of withdrawal, a pair of saline and cocaine pre-exposed group of rats ($n = 6$ each) was sequentially brought to a new place (neither home cage nor locomotor boxes to avoid any confounding factor such as conditioning), and decapitated for western blotting



6. Histology

After completion of the experiments, rats were anesthetized and perfused via intracardiac infusion of saline and 10% formalin. Brains were removed and further post-fixed in 10% formalin. Coronal sections (40 μm) were subsequently stained with cresyl violet for verification of cannula tip placements.

7. Statistics

Total locomotor activity scores (means ± S.E.M.) and Western blotting were analyzed by using two-way ANOVA with injections and groups as factors of variation. *Post-hoc* test were performed by using Scheffé comparisons.

Ⅲ. RESULTS

Experiment 1:

1. Inhibition of ERK phosphorylation in the NAcc blocks the expression of locomotor sensitization to cocaine

Figure 1A shows the locomotor activity counts obtained in both saline and cocaine pre-exposed rats in response to an IP cocaine challenge injection following NAcc pre-infusion of either vehicle or each dose of PD98059 (0.5 and 2.0 μg/side). The two-way between (microinjections)-within (pre-exposures) ANOVA conducted on these data found an approached significance for different microinjections [$F_{2, 32}$=2.95, p<0.068] and multiple significant effects of different pre-exposures [$F_{1, 32}$=35.34, p<0.001], and a microinjections X pre-exposures interaction [$F_{2, 32}$=3.71, p<0.05]. As expected, daily cocaine pre-exposed rats compared to saline, showed a sensitized locomotor activity in response to an IP cocaine challenge injection when vehicle was microinjected into the NAcc (p<0.001, by *post-hoc* Scheffé comparisons). This effect of cocaine, however, was inhibited in a dose-dependent manner by microinjection into the NAcc of PD98059 (p<0.01 in a high dose, 2.0 μg/side, compared to vehicle). In saline pre-exposed rats, PD98059 had no effect on locomotor activity in response to a cocaine challenge indicating that NAcc microinjection of PD98059 produces its effects on sensitized rather than acute locomotor activity by cocaine. The time-course data and location of injection cannula tips in the NAcc of rats that were included in this experiment are illustrated in Figure 1 B and C, respectively.

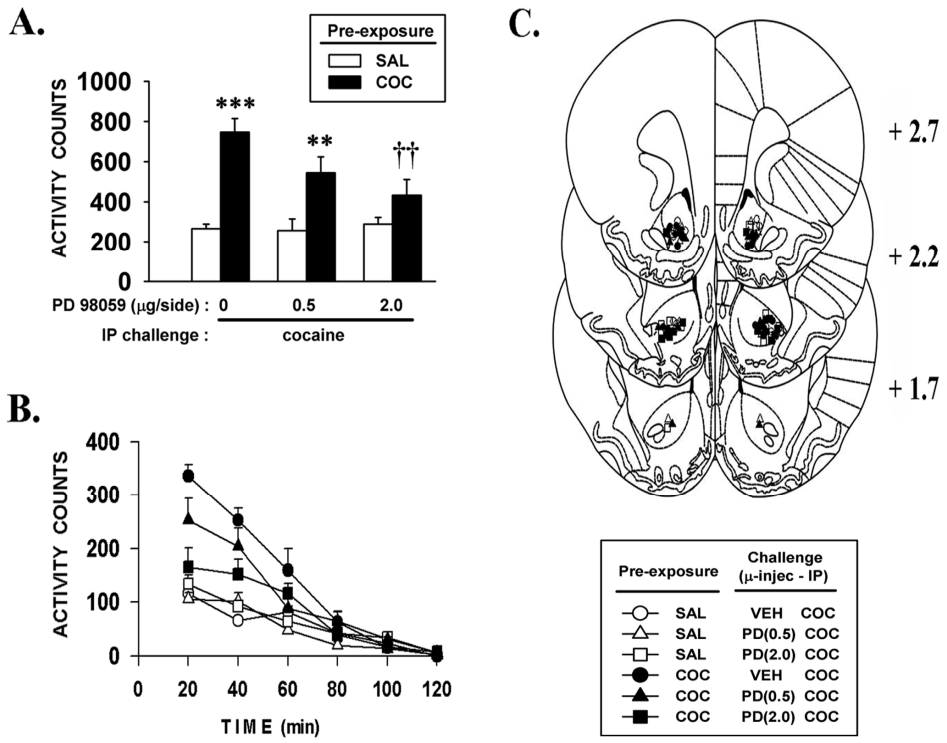

A.
Pre-exposure
SAL
COC
ACTIVITY COUNTS
1000
800
600
400
200
0
***
**
††
PD 98059 (μg/side) : 0 0.5 2.0
IP challenge : cocaine
B.
ACTIVITY COUNTS
400
300
200
100
0
0 20 40 60 80 100 120
TIME (min)
C.
+ 2.7
+ 2.2
+ 1.7
Pre-exposure
Challenge (μ-injec - IP)
SAL VEH COC
SAL PD(0.5) COC
SAL PD(2.0) COC
COC VEH COC
COC PD(0.5) COC
COC PD(2.0) COC

Figure 1. Blockade of ERK phosphorylation by a single microinjection of PD98059 in the NAcc inhibits the expression of cocaine-induced locomotor sensitization. (A) Animals were either saline or cocaine pre-exposed for 6 days. At day 7, their locomotor activity was observed after cocaine (15mg/kg, IP) challenge injection preceded 30 min earlier by acute single bilateral microinjections of either vehicle or PD 98059 (0.5 and 2.0 μg/side) into the NAcc. Data are shown as group mean (+S.E.M.) total locomotor activity counts observed during the first 1 hour. Numbers of rats in each group are 6-7. Symbols indicate significant differences as revealed by *post-hoc* Scheffé comparisons following two-way between (microinjections)-within (pre-exposures) ANOVA. *** $p<0.001$, ** $p<0.01$; significantly more counts in cocaine relative to saline pre-exposed animals. †† $p<0.01$; significant differences in cocaine pre-exposed animals when PD98059 (2.0 μg/side) compared to vehicle was microinjected. (B) Time-course data are shown as group mean (+S.E.M.) locomotor activity counts for every 20 min time-bins obtained during the 2-hr test immediately after cocaine challenge injection. (C) Location of the microinjection cannula tip in the NAcc of rats included in the data analyses. Only rats with injection cannula tips located bilaterally in this site were included no neural damage was observed other than that produced by the insertion of the cannulae. Numbers to the right indicate millimeters from bregma.

2. Acute microinjection of either vehicle or PD98059 alone produced no different locomotor activity compared to saline control.

To further examine the specific ability of NAcc PD98059 to inhibit the expression of locomotor sensitization to cocaine, additional rats were just acute saline challenged following microinjection of either vehicle or PD98059 and their locomotor activity were measured (Fig.2). The one-way ANOVA conducted on these data found no significant differences between groups [$F_{3,16}$=0.07, p<0.976], indicating that either vehicle or any dose of PD98059 used in the present experiments dose not produce the change of basal locomotor activity consistent with previous finding.[18]

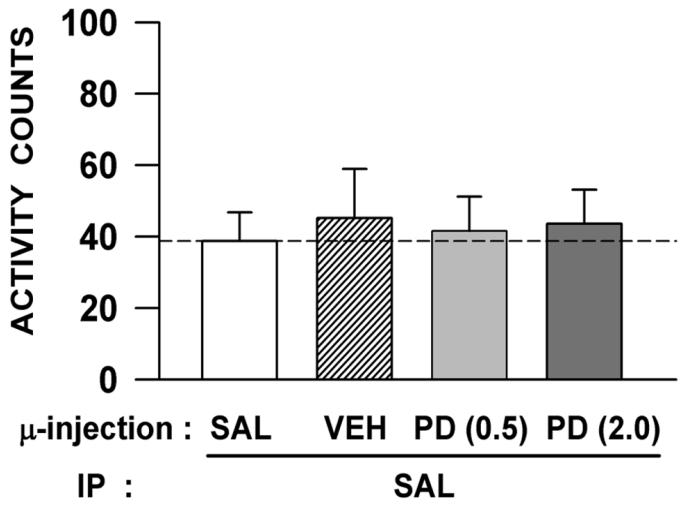


Figure 2. Acute microinjection of vehicle or PD98059 alone into the NAcc produces no different levels of locomotor activity compared to saline control. Locomotor activity is observed following saline (IP) injections preceded 30 min earlier by bilateral microinjections of either saline or vehicle (70% DMSO), PD98059 (0.5 and 2.0 μg/side) into the NAcc. Data are shown as group mean (+S.E.M.) total locomotor activity counts observed for 1 hour (n=5/group).

3. Intra-NAcc microinjection of MEK inhibitor U0126 blocks the expression of locomotor sensitization to cocaine

Figure 3 shows the locomotor activity counts obtained in both saline and cocaine pre-exposed rats in response to an IP cocaine challenge injection following a single NAcc pre-infusion of either vehicle or each dose of U0126 (0.1 and 1.0 μg/side). The two-way between (microinjections) – within (pre-exposures) ANOVA conducted on these data found a significant effect of different pre-exposures [$F_{1,23}$= 6.90, p<0.05]. Again, rats pre-exposed to daily cocaine compared to saline showed a sensitized locomotor activity in response to an IP cocaine challenge injection when vehicle was microinjected into the NAcc (p<0.01, by *post-hoc* Scheffé comparisons). However, this effect of cocaine, similar to PD98059, was inhibited in a dose-dependent manner by a single microinjection into the NAcc of U0126 (p<0.01 in a high dose, 1.0 μg/side, compared to vehicle). In saline pre-exposed rats, U0126 had no effect on locomotor activity in response to a cocaine challenge indicating that NAcc microinjection of U0126 also produced its effect on sensitized rather than acute locomotor activity by cocaine.

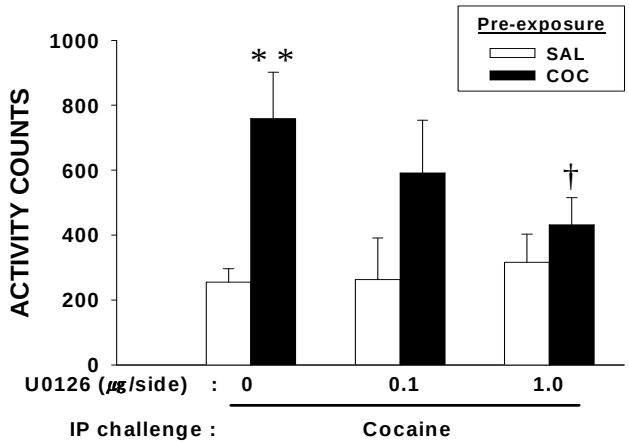


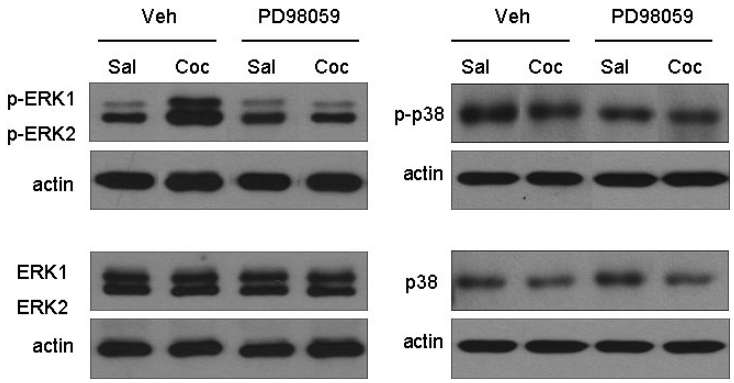
Figure 3. Microinjection of U0126 into the NAcc inhibits the expression of cocaine-induced locomotor-sensitization. Animals were either saline or cocaine pre-exposed for 6 days. At day 7, their locomotor activity was observed after cocaine (15mg/kg, IP) challenge injections preceded 30 min earlier by acute bilateral microinjections of either vehicle or U0126 (0.1 and 1.0 μg/side) into the NAcc. Enhanced increase of locomotor activity in cocaine compared to saline pre-exposed animals was dose-dependently inhibited by a single U0126 microinjection into the NAcc. Data are shown as group mean (+S.E.M.) total locomotor activity counts observed during the 1 hour. Numbers of rats in each group are 4-6. Symbols indicate significant differences as revealed by *post-hoc* Scheffé comparisons following two-way between (microinjection) – within (pre-exposures) ANOVA. ** $p<0.01$; significantly more counts in cocaine relative to saline pre-exposed animals. † $p<0.05$; significant differences in cocaine pre-exposed animals when U0126 (1.0 μg/side) compared to vehicle was microinjected.

Experiment 2:

4. Microinjection of PD98059 into the NAcc selectively inhibits cocaine-induced increase of ERK phosphorylation in this site

Because we used microinjection techniques, in order to confirm the ability of PD98059 to lower phosphorylated ERK 1/2 levels directly in the tissue, the NAcc was taken out from the animals either saline or cocaine IP injected following microinjection of either vehicle or a high dose of PD98059 and their protein levels were analyzed (Fig. 4). Either phosphorylated (p-ERK 1/2 and p-p38) or total (ERK 1/2 and p38) protein kinase levels were examined on separate gels and normalized to β-actin protein levels. The 42-and 44-KDa forms of ERK were quantified together. The increased levels of phosphorylated ERK 1/2 by cocaine in the NAcc were significantly reduced in PD98059 (2.0 μg/side)- compared to vehicle-microinjected animals as revealed by *post-hoc* Scheffé comparisons ($p<0.01$) following two-way between (IP challenges) - within (microinjections) ANOVA (for microinjections [$F_{1, 26}=4.03$, $p<0.055$], IP challenges [$F_{1, 26}=4.33$, $p<0.05$], and a microinjections X IP challenges interaction [$F_{1.26}=4.60$, $p<0.05$]).

A.


Veh
PD98059
Sal
Coc
Sal
Coc
p-ERK1
p-ERK2
actin
ERK1
ERK2
actin
Veh
PD98059
Sal
Coc
Sal
Coc
p-p38
actin
p38
actin

B.

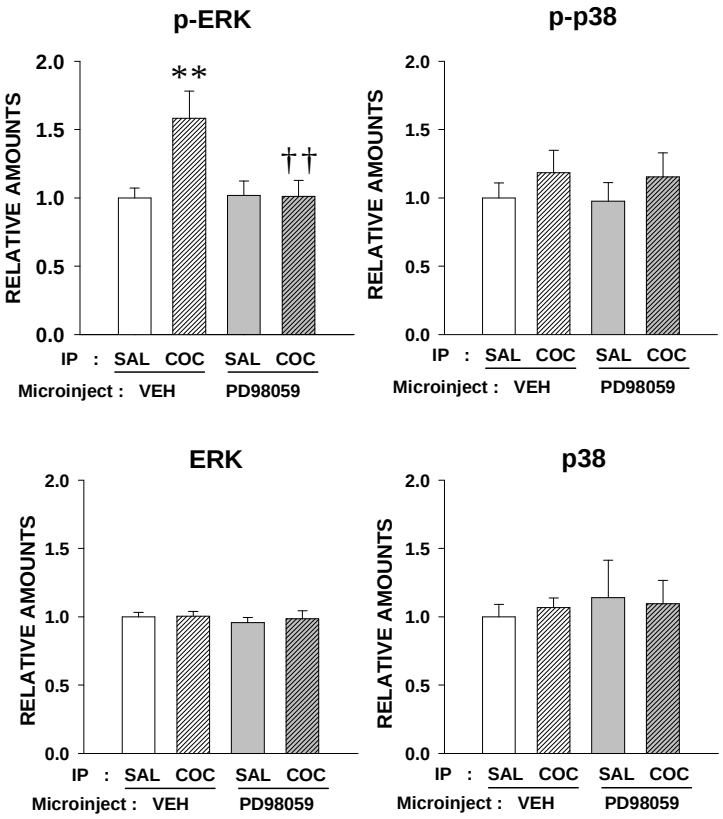

p-ERK
RELATIVE AMOUNTS
2.0
1.5
1.0
0.5
0.0
**
††
IP : SAL COC SAL COC
Microinject : VEH PD98059
p-p38
RELATIVE AMOUNTS
2.0
1.5
1.0
0.5
0.0
IP : SAL COC SAL COC
Microinject : VEH PD98059
ERK
RELATIVE AMOUNTS
2.0
1.5
1.0
0.5
0.0
IP : SAL COC SAL COC
Microinject : VEH PD98059
p38
RELATIVE AMOUNTS
2.0
1.5
1.0
0.5
0.0
IP : SAL COC SAL COC
Microinject : VEH PD98059

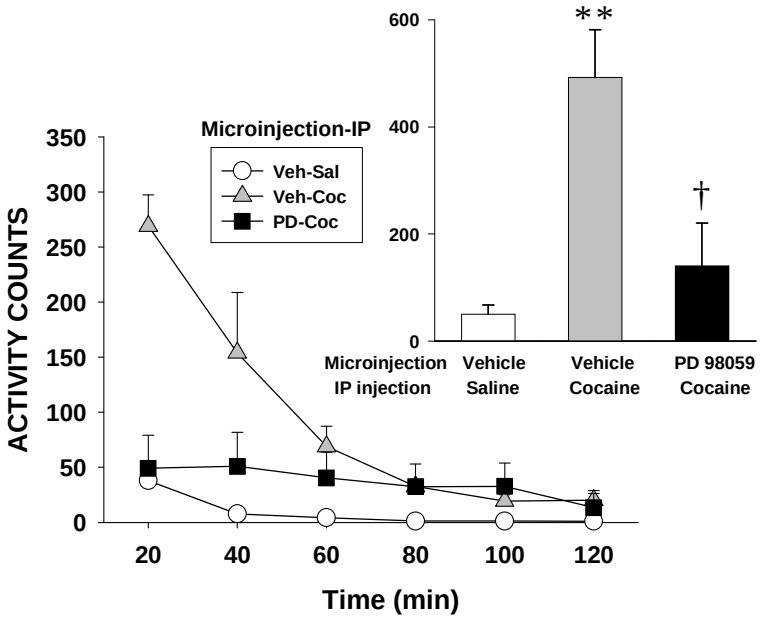
Figure 4. PD98059 in the NAcc lowers cocaine-induced increase of ERK phosphorylation levels in this site. Rats received IP saline or cocaine (15mg/kg) 30 min after a single microinjection of either vehicle or PD 98059 (2.0 μg/side), and then 10 min later, the brains were removed and the NAcc tissues were punched out. (A) Representative Western blots labeled with antibodies against phosphorylated and total ERK1/2, phosphorylated and total p-38, and β-actin. (B) Blots were scanned and the band intensities were quantified using densitometer. Values are normalized to β-actin and expressed as mean (+ S.E.M.) (n=7 to 8/group) transformed to relative amounts of control (vehicle microinjection-saline IP) values. Symbols indicate significant differences as revealed by *post-hoc* Scheffé comparisons following two-way between (IP challenges) -within (microinjections) ANOVA. ** $p<0.01$; significantly higher levels of p-ERK 1/2 in cocaine relative to saline challenged animals. †† $p<0.01$; significant differences in cocaine challenged animals when PD98059 (2.0 μg/side) compared to vehicle was microinjected.

Experiment 3:

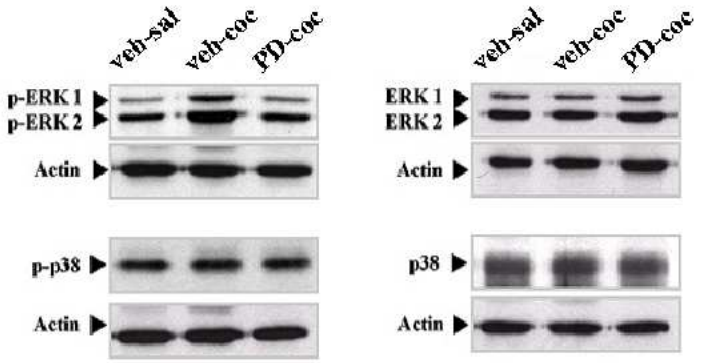
5. Increased locomotor activity when cocaine challenged after 3 weeks of withdrawal in cocaine-sensitized rats, blocked by PD98059

To further examine the ability of NAcc PD98059 to prevent the expression of locomotor sensitization to cocaine in long-term withdrawal, additional cocaine pre-exposed rats were challenged 3 weeks after the last drug pre-exposure. When challenged to cocaine with NAcc PD98059 (2.0 μg/side), rats showed levels of locomotion that were significantly lower than those displayed by cocaine-challenged rats with saline microinjection ($p<0.05$). *Post-hoc* Scheffé comparisons after an ANOVA found significant effects of NAcc infusion [$F_{2, 9}=11.22$, $p<0.01$] (Fig. 5A). Challenge injection of cocaine following vehicle microinjection increased the levels of ERK phosphorylation in the NAcc about 2 times than those obtained by saline challenge (Fig. 5B-C). However, in the presence of NAcc PD98059 (2.0 μg/side), challenged injection of cocaine failed to increase ERK phosphorylation levels in the NAcc. The ANOVA conducted on these data showed significant effects of NAcc infusion [$F_{2, 8}=9.2$, $p<0.01$]. *Post-hoc* Scheffé comparisons indicated that levels were higher only in rats cocaine-challenged with saline microinjection than in other rats ($p<0.05$). The level of total ERK, both phosphorylated and total p38 were all not changed.

A.


Microinjection-IP
Veh-Sal
Veh-Coc
PD-Coc
ACTIVITY COUNTS
350
300
250
200
150
100
50
0
20
40
60
80
100
120
Time (min)
600
400
200
0
**
†
Microinjection
IP injection
Vehicle
Saline
Vehicle
Cocaine
PD 98059
Cocaine

B.


veh-sal
veh-coc
PD-coc
p-ERK 1
p-ERK 2
Actin
p-p38
Actin
veh-sal
veh-coc
PD-coc
ERK 1
ERK 2
Actin
p38
Actin

C.

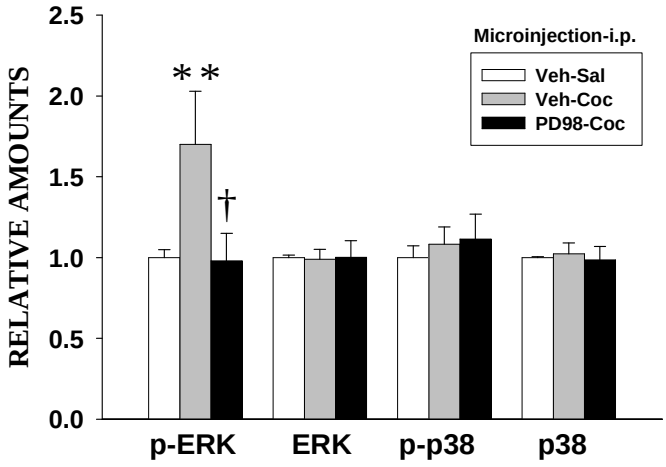


Figure 5. PD98059 in the NAcc blocks the expression of locomotor sensitization to cocaine even when tested after long-term withdrawal. (A) Animals were all cocaine pre-exposed for 7 days with daily IP cocaine injections. After 3 weeks of withdrawal, they were challenged with IP saline or cocaine following 30 min earlier microinjection of either vehicle or PD98059 (2.0 μg/side). Data are shown as group mean (+S.E.M.) locomotor activity counts obtained during the 2 hr test (n=4 for each group) following the sensitization test injection. Inset shows total counts obtained in the first hour of testing. Rats microinjected with saline showed a significantly greater locomotor response to cocaine compared to saline challenge (** $p<0.01$), but this sensitized response was absent when PD98059 was microinjected with cocaine challenge († $p<0.05$). (B) Representative western blots labeled with antibodies against phosphorylated and total ERK, phosphorylated and total p-38, and β-actin. (C) Blots were scanned and the band intensities were quantified using densitometer. Values are normalized to β-actin and expressed as mean (+ S.E.M.) (n=3-

4/group) transformed to relative amounts of control (microinjection saline followed by IP saline as control) values. Symbols indicate significant differences in the levels of p-ERK 1/2 in the NAcc as revealed by *post-hoc* Scheffé comparisons following ANOVA. * $p<0.05$; vehicle-saline vs. vehicle-cocaine; † $p<0.05$; vehicle-cocaine vs. PD98059-cocaine.

Experiment 4:

6. Time-dependent change of ERK phosphorylation levels in the NAcc during withdrawals from repeated cocaine

Table 1 shows the locomotor activity counts obtained in rats pre-exposed to cocaine or saline. Only rats that showed more than 20% increase of locomotor activity on day 7 relative to day 1 were considered for further analyses. This procedure is commonly used for the development of sensitization by cocaine. Of the 32 rats prepared for cocaine sensitization, 8 were dropped for failing to meet this criterion.

Any protein levels measured on the next day following pre-exposures were not different in cocaine compared from saline pre-treated rats (Fig. 6). Paired *t*-test comparisons of these data revealed no significant differences ($p < 0.97–0.26$). Fig. 7A shows the representative Western blots labeled with different antibodies in either saline or cocaine pre-exposed groups following longer withdrawal periods (7, 14 or 21 days). The band intensities on the blots were quantified and expressed as relative amounts of control (saline pre-exposed group at withdrawal day 7) values (Fig. 7B). The ANOVA conducted on these data found significant effect of different pre-exposure groups [$F_{1, 30}=8.24$, $p<0.01$] only in p-ERK1/2 levels. *Post hoc* Scheffé comparisons revealed significant differences ($p<0.01$) in the levels of p-ERK1/2 in cocaine compared with saline pre-exposed group at withdrawal day 7. Interestingly, this effect is gradually diminished in a time-dependent manner following withdrawal periods ($p<0.05$, withdrawal day 21 compared with day 7 in cocaine pre-exposure). Total ERK1/2 and both phosphorylated and total p38 levels showed no differences between groups. These results indicate that the basal ERK1/2 phosphorylation levels in the NAcc differentially change following withdrawal periods from cocaine pre-exposures.

Table 1. Locomotor activity counts during pre-exposures

Pre-exposure[a]	**Day 1**	**Day 7**
Saline (24)	36 ± 7	54 ± 8
Cocaine (24)	222 ± 50	600 ± 55

Data are shown as group mean (±S.E.M.) locomotor activity counts. All rats were habituated for 1 h and their locomotor activity measured for an additional 1 h following their respective injections. Only at day 1 and 7, locomotor activity was measured during the pre-exposure injections (once daily total 7 injections).
[a] Numbers in parentheses indicate *n* per group.

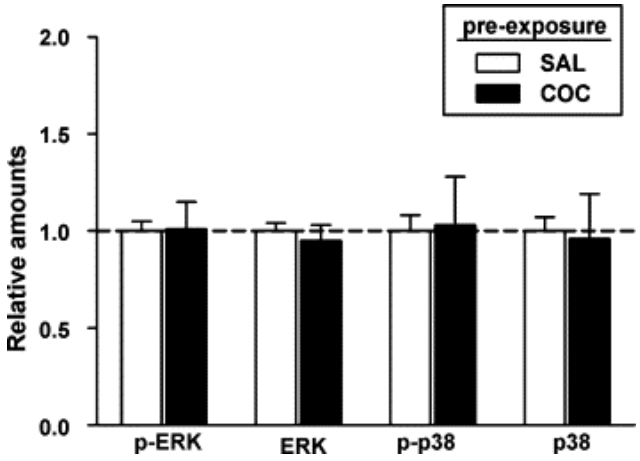
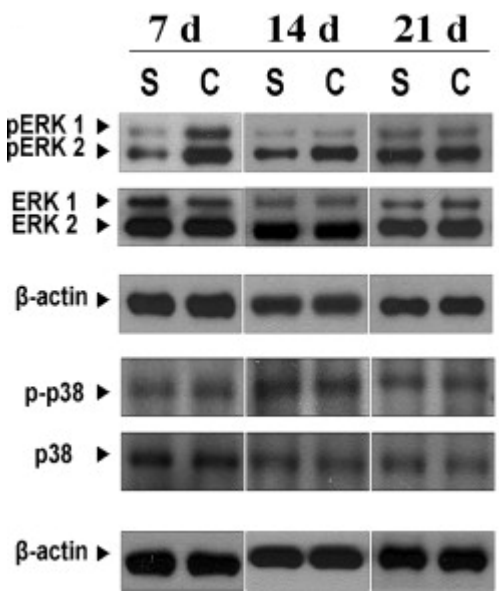


Figure 6. The basal levels of ERK phosphorylation in the NAcc are not different in cocaine compared from saline pre-exposed groups on withdrawal day 1. Animals were pre-exposed to daily IP injections of cocaine or saline for 7 days. On withdrawal day 1, brains were removed and the NAcc was punched out. Western blots were scanned and the band intensities were quantified using densitometer. Values are normalized to β-actin and expressed as mean (+S.E.M.) (n = 6 per group) transformed to relative amounts of control values (saline pre-exposed group as control). Total ERK1/2 and both phosphorylated and total p38 levels also showed no differences between groups.

A.


7 d
14 d
21 d
S C S C S C
pERK 1
pERK 2
ERK 1
ERK 2
β-actin
p-p38
p38
β-actin

B.

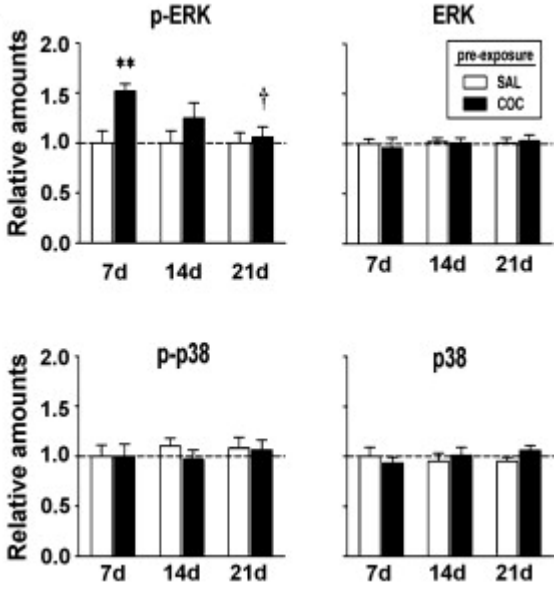

p-ERK
ERK
pre-exposure
SAL
COC
Relative amounts
2.0
1.5
1.0
0.5
0.0
**
†
7d
14d
21d
p-p38
p38

Figure 7. The basal levels of ERK phosphorylation in the NAcc change following withdrawal period from cocaine pre-exposures. Animals were pre-exposed to daily IP injections of cocaine or saline for 7 days. On each of withdrawal day 7, 14, and 21, their brains were removed and the NAcc was punched out. (A) Representative Western blots labeled with antibodies against phosphorylated and total ERK1/2, p38, and β-actin. Groups indicate either saline (S) or cocaine (C) pre-exposures with different withdrawal periods. (B) Blots were scanned and the band intensities were quantified using densitometer. Values are normalized to β-actin and expressed as mean (+S.E.M.) (n=6 per group) transformed to relative amounts of control values (saline pre-exposed group at withdrawal day 7 as control). Symbols indicate significant differences on the levels of p-ERK1/2 in the NAcc as revealed by post hoc Scheffé comparisons following two-way ANOVA. $^{**}p<0.01$, saline compared with cocaine pre-exposure at withdrawal day 7; $^{\dagger}p<0.05$, withdrawal day 21 compared with day 7 in cocaine pre-exposure. Total ERK1/2 and both phosphorylated and total p38 levels showed no differences between groups.

IV. DISCUSSION

The present results demonstrate that a direct bilateral microinjection into the NAcc of PD98059, or U0126, MEK inhibitor, dose-dependently inhibits the expression of cocaine-induced behavioral sensitization. This effect was clearly observed when challenged by cocaine following the acute single microinjection of PD98059, or U0126, in cocaine pre-exposed animals, while it was not in saline pre-exposed. Microinjection of PD98059 into the NAcc produced no effect on the basal locomotor activity, while it specifically lowered cocaine-induced increase of phosphorylated ERK level in this site. These results illustrate the importance of ERK activation in the NAcc for the expression of cocaine-induced behavioral sensitization. Further, the basal levels of ERK1/2 phosphorylation in the NAcc change in a time-dependent manner during the drug-free withdrawal periods in cocaine compared to saline pre-exposed rats. These findings are the first systematic demonstration, to our knowledge, that ERK1/2 phosphorylation levels in the NAcc are differentially regulated during drug-free withdrawal periods after repeated cocaine pre-exposures.

It has recently been shown that the development of sensitization to cocaine is prevented by systemic pre-treatment of a MEK inhibitor, SL327, prior to each drug administration during the induction phase.[9, 15] These effects were further supported by earlier results that the initiation of behavioral sensitization to cocaine was blocked by intra-VTA microinjection of another MEK inhibitor, PD98059.[7] These results indicate that consistent ERK activation, especially in the VTA, during the induction phase is necessary for the development of behavioral sensitization by cocaine. However, the role of ERK activation in the expression of behavioral sensitization, which is mediated through a different neural substrate (i.e., the NAcc),[13, 14] remained undefined. Although recent results also showed that the blockade of ERK pathway did not alter the expression of behavioral sensitization to cocaine and amphetamine,[9] these results were all obtained with systemic injection of SL327. This drug crosses

the blood-brain barrier and reduced ERK phosphorylation levels in various brain regions,[9] which thereby makes it unclear whether the NAcc ERK actually contributes to the expression of behavioral sensitization to psychomotor stimulants. In the present results, we used a single direct microinjection into the NAcc of different types of MEK inhibitors, PD98059, or U0126, and found that ERK activation specifically in this site is actually necessary for the expression of behavioral sensitization. The NAcc exists as a central part of a complicate neuronal circuit mediating rewarding and motor behavior and its activity is regulated by signals coming from other brain regions. The difference in our results compared from previous finding that SL327 have no effect on the expression of behavioral sensitization may have come out because we inhibited ERK phosphorylation directly in the NAcc, whereas others inhibited it simultaneously in many brain regions causing its effect in the NAcc to be compromised by signals coming from other regions.

The delivery vehicle we used for MEK inhibitors was DMSO. Microinjection of this vehicle alone (up to 70% for PD98059) compared to saline didn't affect basal locomotor activity in response to IP saline injection. Similar to and consistent with our findings, others have also reported that various dose ranges (50-100%) of DMSO were microinjected into the NAcc as well as into the VTA without evident disturbances of locomotor activity and neuronal damage.[7, 12, 18] Our present results also showed that microinjection into the NAcc of PD98059 had no effect on the locomotor activity produced by either acute cocaine (saline pre-exposed and cocaine challenged in Fig. 1A) or saline injection (Fig. 2). These results indicate that the inhibitory effect of PD98059 was rather specific to the expression of locomotor sensitization to cocaine and further support and extend its role in the regulation of neuronal plasticity induced by drugs of abuse found in various aspect of addiction. [9, 10, 12, 15, 18]

It has been reported that the levels of ERK phosphorylation in the NAcc increased by cocaine challenge.[12] In order to verify that this increase of cocaine-induced ERK phosphorylation levels is actually lowered by our microinjection

procedure, we measured protein levels in both the NAcc and dorsal striatum after either vehicle or PD98059 microinjection. Although a recent finding using knockout mice suggest that ERK 1 and 2 may have different roles in cocaine-induced plasticity,[15] it remains to be further explored, so that we decided in the present experiments to quantify both proteins together. As shown in Fig. 4, our microinjection procedure specifically lowered cocaine-induced increase of phosphorylated ERK 1/2 levels in the NAcc, but not affected the levels of total ERK 1/2 and another MAP kinase, p38, both phosphorylated and total. These results support that our behavioral finding were actually obtained correlated with the specific blockade of ERK phosphorylation in the NAcc and further suggest that specific ERK activation in this site may play an important role in neuronal plasticity contributing to the expression of sensitization.

Evidence shows that the interaction of both dopamine and glutamate in the NAcc is necessary for the expression of behavioral sensitization by psychomotor stimulants[19, 20, 21] and previous exposure to these drugs increases dopamine and glutamate overflow in the NAcc.[22, 23] Interestingly, it has been shown that cocaine-induced ERK activation is also dependent on both dopamine and glutamate activation.[8, 24] Together with our present findings, this leads to the hypothesis that the increase of dopamine and glutamate levels by cocaine challenge may subsequently result in increase of ERK phosphorylation, and thereby contributes to the expression of behavioral sensitization. However, it remains to be further explored.

It has been previously shown in laboratory animals that cocaine craving progressively incubates during drug-free withdrawal periods.[25] Associated with this phenomenon, it has also been found that BDNF protein levels in the NAcc as well as in other brain regions involved in drug addiction progressively increase after cocaine withdrawal.[26] In parallel with these, more recent research has shown that the cell surface expression of AMPA receptors in the NAcc increases during 7–21 days, but not 1 day, of withdrawals from cocaine pre-exposures.[16, 17] These results indicate that the drug-free withdrawal periods are

not dormant but rather actually regulate the expression of molecules by which they may contribute to the long-term expression of behavioral sensitization and subsequently of craving. We have found in the present results that the basal levels of ERK1/2 phosphorylation are also differentially regulated during the drug-free withdrawal periods after cocaine pre-exposures. They show no changes on a short withdrawal (day 1) but significant increases on an intermediate withdrawal (day 7), suggesting that ERK1/2 may have a potential role for setting a stage during intermediate withdrawal period for subsequent consolidation of drug craving and behavioral sensitization. It is interesting to find some similar time-courses for ERK1/2 in our data with AMPA receptors in others showing that the cell surface expression of them in the NAcc increases during 7–21 days, but not 1 day, of withdrawals from cocaine pre-exposures. Also consistent with our findings, it has recently been shown that ERK2 phosphorylation levels in the NAcc increase to the similar levels of ours on withdrawal day 14 from cocaine pre-exposures.[16] Our present findings, however, further show that ERK1/2 phosphorylation levels gradually lowered from withdrawal day 7 through 21, which is interesting compared to previous reports that BDNF gets gradually increased and AMPA cell surface expression keeps increased levels during these periods.[16, 17, 26] We do not know yet how they are differentially regulated during withdrawals. However, it is very suggestive that differential activation of ERK1/2 in the NAcc during the withdrawals may be linked to those other molecules in terms of contribution to the incubation of craving as well as the long-term expression of behavioral sensitization, considering the fact that activation of ERK pathway involves in both AMPA and BDNF-activated receptors trafficking.[17, 27]

Evidence indicates that ERK pathway is importantly involved in long-term adaptive processes by cocaine.[28] In the extension of this notion, our present results further revealed that ERK1/2 phosphorylation levels in the NAcc are differentially regulated during the cocaine withdrawal periods, suggesting that time-dependent phosphorylation changes may contribute to the incubation of

cocaine craving.

V. CONCLUSION

The present results indicate that the blockade of ERK activation in the NAcc by direct bilateral microinjection into this site of PD98059 or U0126 dose-dependently inhibits the expression of cocaine-induced behavioral sensitization, while acute microinjection of either vehicle or PD98059 alone produces no effect on the basal locomotor activity compared to saline control. These findings illustrate the importance of ERK 1/2 activation in the NAcc for the expression of cocaine-induced behavioral sensitization. Evidence indicates that ERK pathway is importantly involved in long-term adaptive processes by cocaine. In the extension of this notion, our present results further revealed that ERK1/2 phosphorylation levels in the NAcc are differentially regulated during the cocaine withdrawal periods, suggesting that time-dependent phosphorylation changes may contribute to the incubation of cocaine craving.

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Abstract (in Korean)

Nucleus accumbens 에서의 Extracellular signal-regulated kinase signaling 과 코카인에 의한 행동과민반응

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김 승 우

코카인을 반복적으로 주입하면 행동과민반응이 일어나게 된다. Mitogen-activated protein (MAP) kinases 는 세포성장과 분화, 신경가소성등에 중요한 역할을 한다. MAP Kinases중 하나인 extracellular signal-regulated kinase (ERK)는 여러 신경생물반응에 관련이 있는 것으로 알려지고 있다. 비록 nucleus accumbens (NAcc)는 행동과민반응의 발현을 매개하는 곳으로 알려지고 있지만 이곳에서 ERK의 정확한 역할에 대해서는 아직 알려지지 않고 있다. 본 연구에서는 6일간 코카인(15mg/kg)을 복강주입하고 7일째 PD98059 (0.5 또는 2.0㎍/side) 또는 U0126 (0.1 또는 1.0㎍/side)을 직접 NAcc에 microinjection하여 ERK인산화를 저해하였을 때 코카인에 의한 행동과민반응이 농도의존적으로 저해되는 것을 증명하였다. 이러한 효과는 3주간의 withdrawal기간이 지난 후에도 나타났다. Vehicle 또는 PD98059만을 microinjection하였을 때에는 saline을 주입한 쥐와 비교했을 때에는 행동반응에 아무런 차이가 없었다. PD98059 (2.0㎍/side)를 NAcc에 microinjection하였을 때 코카인에 의한 ERK인산화는 저해되었지만, p38에는 아무런 영향을 주지 않았다.

더 나아가 NAcc에서 ERK인산화가 코카인 주입 withdrawal시간

별로 어떻게 변하는지에 대해 알아보았다. ERK 인산화의 basal level은 1일간의 withdrawal에서는 아무런 변화가 나타나지 않았지만, 7일째에는 증가되어 나타났고, 점점 감소하여 21일째에는 saline 주입 쥐와 같은 수준으로 감소되었다. 전체 ERK와 p38은 시간대별로 아무런 변화가 나타나지 않았다. 이러한 결과들을 종합하여 보았을 때 NAcc의 ERK 인산화는 코카인에 의한 행동과민반응의 발현에 필수적인 역할을 하는 것으로 생각된다. 또한 시간대별 ERK인산화의 변화는 약물의 갈망과 같은 영구적인 행동의 변화에 기여할 것으로 생각된다.

핵심 되는 말: 코카인, ERK, 행동과민반응, nucleus accumbens, microinjection, 갈망

Publication list

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