Amphetamine regulates ezrin-radixin-moesin proteins signaling in the nucleus accumbens core via glycogen-synthase-kinase 3β

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Directed by Professor Jeong-Hoon Kim

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Amphetamine regulates ezrin-radixin-moesin proteins signaling in the nucleus accumbens core via glycogen-synthase-kinase 3β

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Psychomotor stimulants such as cocaine or amphetamine (AMPH) produce the structural changes in the nucleus accumbems by increasing dendritic spine density after developments of behavioral sensitization and self-administration. The ezrin-radixin-moesin (ERM) proteins have been implicated not only in cell-shape determination but also in signaling pathway. However, it has not been determined yet how ERM proteins are regulated by AMPH. Here I show that the phosphorylation levels of ERM proteins are time-dependently

decreased in the NAcc core, but not in the shell, by a single injection of AMPH (2 mg/kg, IP). I also found a similar time-dependent effect of AMPH on the phosphorylation levels for Akt and GSK3β in this site. When rats were co-administered with LiCl (100 mg/kg, IP), a GSK3 inhibitor, and either saline or AMPH (2 mg/kg, IP), and the phosphorylation levels for both GSK3β and ERM in the NAcc core were significantly recovered by this treatment. Further, I show that S9 peptide (0.5 or 5.0 μg/μl), GSK activator, decreases both pGSK and pERM levels in this site. Further, microinjection into the NAcc core of LY294002 (0.4 or 4.0 μg/μl), PI3 kinase inhibitor, decreases, whereas bpV (0.5 or 5.0 μg/μl), its activator, increases pERM levels in this site. Together, these results suggest that AMPH reduces pERM levels in the NAcc core via GSK3β.

It is well-known that AMPH pre-exposure produces locomotor sensitization when challenged with the same drug after a certain period of withdrawal. Here I show that chronic AMPH-induced behavioral sensitization reduces the basal levels of pERM as well as pAkt and pGSK proteins in the same direction in the NAcc core, suggesting that chronic AMPH may down-regulate pERM levels

through PI3 kinase-Akt-GSK signaling pathways in a longer-lasting fashion contributing to produce a more endurable biochemical changes leading to addicted behaviors.

Key words: amphetamine, ERM, Akt, GSK3β, nucleus accumbens core, behavioral sensitization, lithium, S9 peptide, bpV, LY294002

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I. INTRODUCTION

Drugs abuse is a chronically relapsing disorder that is characterized by compulsive drug seeking and taking[1]. It is known to be mediated by rewarding circuit composed of several brain regions including ventral tegmental area (VTA), nucleus accumbens (NAcc), and prefrontal cortex (PFC).[2] Among them, the NAcc is an important neuronal substrate mediating the effects of drugs of abuse. It has been shown that psychomotor stimulants like cocaine or amphetamine (AMPH) produce the structural changes in this site by increasing dendritic spine density after developments of behavioral sensitization and self-administration.[3-6] Interestingly, the NAcc has 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 psychomotor stimulants induce 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 thesis, it has been sought to determine whether a single acute AMPH 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. This characteristic makes ERM proteins play important roles in many cellular processes including microvilli formation, motility, cell adhesion, cytokinesis and integration of membrane transport with signaling pathways.[11,12] The ERM proteins, prior to activation, exist in an auto-inhibited conformation in which the C-terminal domain binds and masks the N-terminal domain in the cytoplasm.[13] In order to stably maintain F-actin binding and consequently its linkage 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, which then regulates dendritic spine morphology.[11] Interestingly, it has been recently shown that the phosphorylation levels of ERM proteins in the NAcc are decreased in a time- and dose-dependent manner by acute

injection of cocaine,[14] suggesting that psychomotor stimulant drugs like cocaine is able to initiate possible cellular events leading to actin cytoskeletal remodeling by regulation of ERM signaling in this site.

It was previously shown that microinjection into the NAcc of the phosphoinositide 3-kinase (PI3 kinase) inhibitor, LY294002, blocks the expression of cocaine-induced behavioral sensitization.[15] Further, protein kinase B (Akt) and glycogen synthase kinase 3β (GSK3β), downstream targets of PI3 kinase, are known to involve in dopamine-related signaling pathways and accordingly psychomotor stimulant-induced characteristic behaviors.[16] These results strongly suggest that PI3 kinase-Akt-GSK3β signaling pathways relevantly contribute to the expression of psychomotor stimulants-induced addicted behaviors. Classically, the functions of dopamine receptors have been associated with the regulation of cAMP-PKA (protein kinase A) through G-protein-mediated signaling.[3,17,18] Dopamine receptors constitute five different receptors and divide into D1-like (D1 and D5) and D2-like (D2, D3 and D4) subtypes. The D1-like receptors couple mostly to $G\alpha_s$ and stimulate the production of the second messenger cAMP and the activity of PKA, which is known as canonical DA signaling pathway and plays important role in the neuronal plasticity.[3,19-21] On the other hand, recent investigations have shown that dopamine receptors exert their effects through cAMP-independent mechanisms. Activated D2-like receptors can function through an Akt-GSK3 signaling cascade. This new dopamine receptor signaling displays different kinetic properties.[16,22,23]

Akt is a serine/threonine protein kinase that functions as a critical regulator of cell survival and proliferation.[24] Akt contains two regulatory phosphorylation sites, Thr308 and Ser473. Activation of Akt by growth

factors involves a PI3 kinase dependent membrane translocation step, followed by phosphorylation of two key regulatory sites.[24] Thr308 is phosphorylated by the phosphoinositide-dependent kinase-1 (PDK1) and the other phosphorylation site, Ser473, can be autophosphorylated or phosphorylated by a distinct kinase such as PDK2.[25-27] Phosphorylation of Thr308 partially activates Akt, while phosphorylation of both sites is required for full activation. Significantly, phosphorylation of Ser473 alone has little effect on Akt activity.[28] A major downstream of Akt is GSK3.[6] It is identified originally as a regulator of glycogen metabolism, but is now known to regulate a diverse array of cell functions, including cell division, proliferation, differentiation, apoptosis, and microtubule function.[29,30] It is also a seine/threonine kinase and exists as two forms of subtypes: GSK-3α and GSK-3β that is 51 and 47kDa, respectively, and share almost complete sequence identity in their protein kinase domains but differ in other regions.[31] These enzymes are highly regulated by phosphorylation. Activated Akt inhibits GSK3 by phosphorylating the Ser9 residue of GSK3β or Ser21 residue on GSK3α, which are located in their regulatory N-terminal.[32] In mammals, both isoforms of GSK3 exist. Of the two, especially, GSK-3β is highly expressed in brain including the amygdala, NAcc, and hippocampus.[33,34] And it has been shown to be involved in the dopamine -associated behaviors and in the synaptic plasticity.[35-37]

Interestingly, these PI3 kinase signaling pathways have been shown to regulate ERM phosphorylation levels in neuronal cell cultures.[38] Even with their important role in cell shape determination and relevant signaling pathways, how ERM proteins and PI3 kinase-Akt-GSK3β signaling pathways are inter-related in the NAcc, especially in association

with psychomotor stimulant drugs, have not been studied at all yet. Further, increasing knowledge indicate that the NAcc has two different sub-regions, the core and the shell, and they have differential functional role in addiction-related behaviors.[39,40] Thus, it has been examined in the present thesis how AMPH, either a single or chronic injection, regulates the phosphorylation levels of ERM proteins and PI3 kinase-Akt-GSK3β signaling pathways in the NAcc core and shell.

II. MATERIALS AND METHODS

1. Subjects and surgery

Male Sprague-Dawley rats weighing 220-260 g on arrival were obtained from Orient Bio Inc. (Seongnam-si, Korea). They were housed three per cage in a 12-h light/dark cycle room (lights out at 7:00 pm) and all experiments were conducted during the day time. Rats had access to food and water *ad libitium* at all times. During the surgical operation, rats were anesthetized with ketamine (100 mg/kg, IP) followed by xylazine (6 mg/kg, IP), 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 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 allowing full recovery for 5 days at least. All animal use procedures' were conducted according to an approved Institutional Animal Care and Use Committee protocol.

2. Drugs

D-Amphetamine sulfate (United States Pharmacopeial Convention, Inc., Rockville, MD, USA), lithium chloride (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in sterile 0.9% saline. S9 peptide was obtained as a gift from Dr. Soo Young Lee's laboratory at Ewha Women's University. PI3 kinase activator, bpV (PEPTRON, Daejon, Korea), and, its inhibitor, LY 294002 hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 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 0.5 or 5.0 μg/μl in saline (S9 peptide and bpV), and of 0.4, 4.0 μg/μl in 80% DMSO (LY294002).

3. 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 of 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.

4. Brain tissue preparation

For Figure 1 and 2 experiments, animals were decapitated at different time points (0, 15 and 60 min) after saline or AMPH IP injections. For all other experiments, animals were decapitated 60 min after either IP or microinjections. Brains were rapidly removed and coronal sections (1.0 mm thick extending 1.6 – 2.60 mm from bregma) were obtained with an ice-cold brain slicer. Tissue punches (1.0 mm diameter) were obtained in the NAcc core and shell regions 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.

5. 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), phosphor-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), total Akt (1:10,00 dilution in PBS-T with 5 % skim milk; Cell Signaling, Beverly, MA, USA), phosphor-Akt (specific to detect phosphorylated Akt at threonine 308; 1:500 dilution in PBS-T with 5 % bovine serum albumin; Cell Signaling, Beverly, MA, USA), total GSK3 (1:10,000 dilution in PBS-T with 5 % skim milk; Cell Signaling, Beverly, MA, USA) and phosphor-GSK3β (specific to detect phosphorylated GSK3β at serine 9; 1:1,000 dilution in PBS-T with 5 % bovine serum albumin; Cell Signaling, Beverly, MA, USA) 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 (AbFrontier, Seoul, Korea) 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^{TM} Western Blot Stripping Buffer (Pierce, Rockford, IL) and re-probed with anti-β-actin (1:10,000 dilution in PBS-T with 5 % skim milk; Abcam, Cambridge, UK).

6. Behavioral sensitization model

Figure 1. Schedule of behavioral sensitization model.

The experiments consisted of a pre-exposure phase and a test phase for sensitization. For pre-exposure, rats were administered with either saline or AMPH (1 mg/kg, IP) on four occasions, one injection every 2-3 days. This regimen of AMPH injection is known to produce enduring sensitization of the locomotor response to AMPH[41,42]. Immediately after the first and fourth injections, rats were placed in the activity boxes and their locomotor activity was measured for 1 h. During other injections, locomotor activity was not measured and animals following these injections were returned to their home cages. Test for sensitization was made two weeks after the last exposure injection. Rats were first habituated to the activity boxes for 30 min and then injected AMPH (1 mg/kg, IP) and immediately their locomotor activity were measured for an additional 1 h.

7. Locomotor activity

Locomotor activity was measured in a bank of 6 activity boxes (25 x 35 x 40 cm) (IWOO Scientific Corporation, Seoul, Korea) that were 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 photo beams (Med Associates, St. Albans, VT, USA) positioned 4.5 cm above the floor and spaced evenly along the longitudinal axis of each box, estimated horizontal locomotion. Two additional photo beams, positioned on the sidewall 14.5 cm above the floor and 7.5 cm from the front and back walls, estimated rearing.

8. Statistics

The data were analyzed with either one-way or two-way ANOVA (analysis of variance) followed by either post-hoc Bonferoni comparisons or Student-Newman-Keuls comparisons. Differences between experimental conditions were considered statistically significant when $p<0.05$.

III. RESULTS

1. AMPH decreases the phosphorylation levels of ERM proteins in the NAcc core

In order to test whether AMPH may regulate ERM proteins in the NAcc, the ratio of the phosphorylated to total ERM protein levels was examined by immunoblotting method with the NAcc tissues (core and shell separated) obtained at two different time points (15 and 60 min) after a single intraperitoneal (IP) injection of AMPH (2 mg/kg) (Fig. 2). Post-hoc Bonferroni comparisons after ANOVA ($F_{2,15} = 9.15$; $p < 0.003$) conducted on these data revealed that the ratio of the phosphorylated to total ERM was significantly reduced ($p < 0.05 - 0.01$) in AMPH compared to saline administered rats at between 15 and 60 min time points measured (Fig. 2).

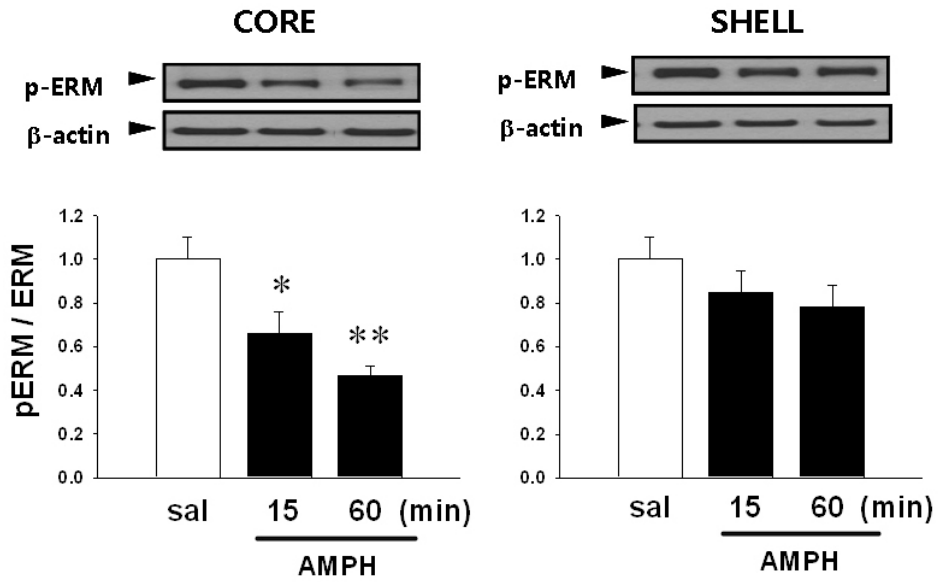


Figure 2. The change of phosphorylation levels of ERM proteins by amphetamine in the NAcc core and shell. Representative Western blots were shown. Values for the band intensities normalized to β-actin was expressed as mean + s.e.m. relative to saline control. Symbols indicate significant differences as revealed by *post-hoc* Bonferroni comparisons following one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, ** $p < 0.01$; significantly different from saline (n=6 per group).

2. AMPH time-dependently decreases both pAkt and pGSK levels in the NAcc core

Next, both pAkt and pGSK levels were examined in two sub-regions of the NAcc after AMPH injection. When brain tissue samples were obtained at both 15 and 60 min after a single AMPH (2 mg/kg, IP) injection, the ratio of the phosphorylated form of Akt to the total protein was significantly decreased in AMPH compared to saline injected rats at 60 min only in the core (ANOVA, $F_{2,17} = 3.87$; $p < 0.05$), while the ratio of the phosphorylated form of GSK to the total protein was significantly decreased in both the core and the shell (ANOVA, $F_{2,17} = 8.74$; $p < 0.003$ and $F_{2,17} = 4.23$; $p < 0.05$, for the core and the shell, respectively) (Fig. 3). These results indicate that AMPH may regulate both Akt and GSK signaling in the same direction with ERM proteins resulting in activation of GSK proteins in the NAcc core.

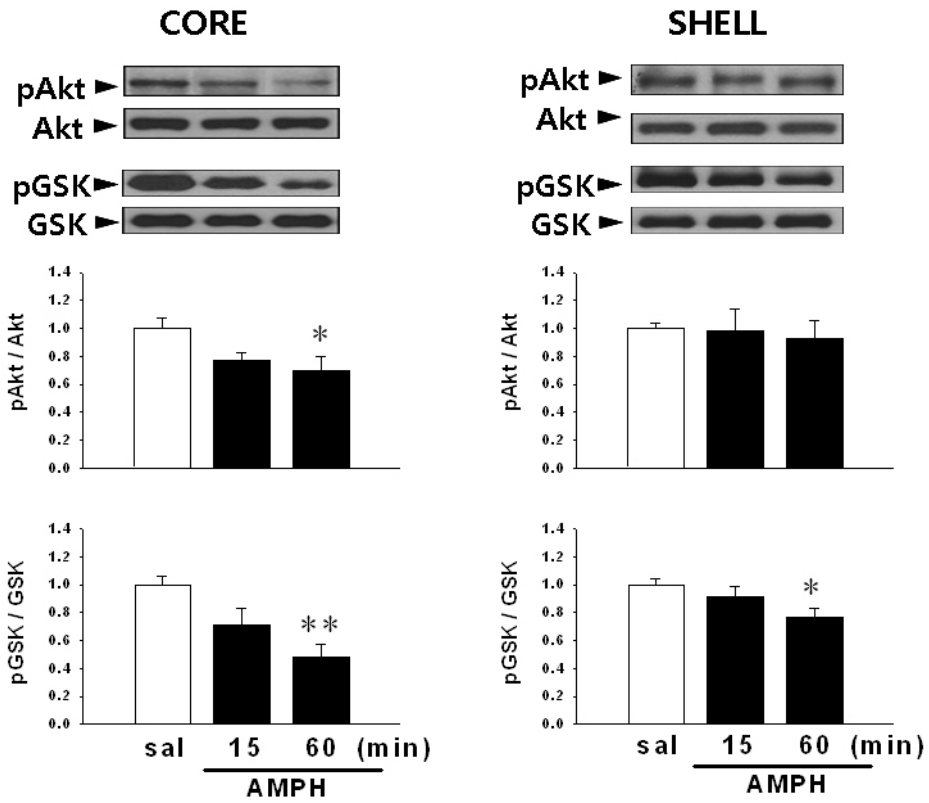


Figure 3. The change of phosphorylation levels of pAkt and pGSK by amphetamine in the NAcc core and shell. Representative western blots were shown. Values for the band intensities were expressed as mean + s.e.m. relative to total proteins. Symbols indicate significant differences as revealed by *post-hoc* Bonferroni comparisons following one-way ANOVA. * $p < 0.05$, ** $p < 0.01$; significantly different from saline (n=6 to 8 per group).

3. Lithium dose-dependently recovers the decreased phosphorylation levels of GSK and ERM proteins in the NAcc core, but not in the shell

In order to test whether GSK activation contributes to the decrease of pERM levels in the NAcc core, a well-known GSK inhibitor lithium (50 and 100 mg/kg, IP) was administered to rats together with either saline or AMPH, and phosphorylated GSK and ERM levels were measured in both the core and the shell. Interestingly, it was found that lithium dose-dependently recovered the ratio of phosphorylated levels to total GSK and ERM proteins only in the core, but not in the shell (Fig. 4). Two-way between-within ANOVA with lithium doses as between and drug as the within factors revealed significant effects of drug for both GSK and ERM ($F_{1,28} = 4.58$; $p < 0.05$, and $F_{1,18} = 21.98$; $p < 0.001$, respectively). It also revealed significant effect of lithium doses x drug interactions ($F_{2,18} = 4.51$; $p < 0.05$) for ERM proteins. Only in the core, AMPH exposed rats showed a significant recovery of pERM levels in lithium high dose (100 mg/kg, IP) compared to vehicle control rats ($p < 0.05$; as revealed by post hoc Bonferroni comparisons). These results suggest that AMPH-induced decrease of phosphorylated ERM levels is under the regulation of GSK signaling as its upstream effectors in the NAcc core.

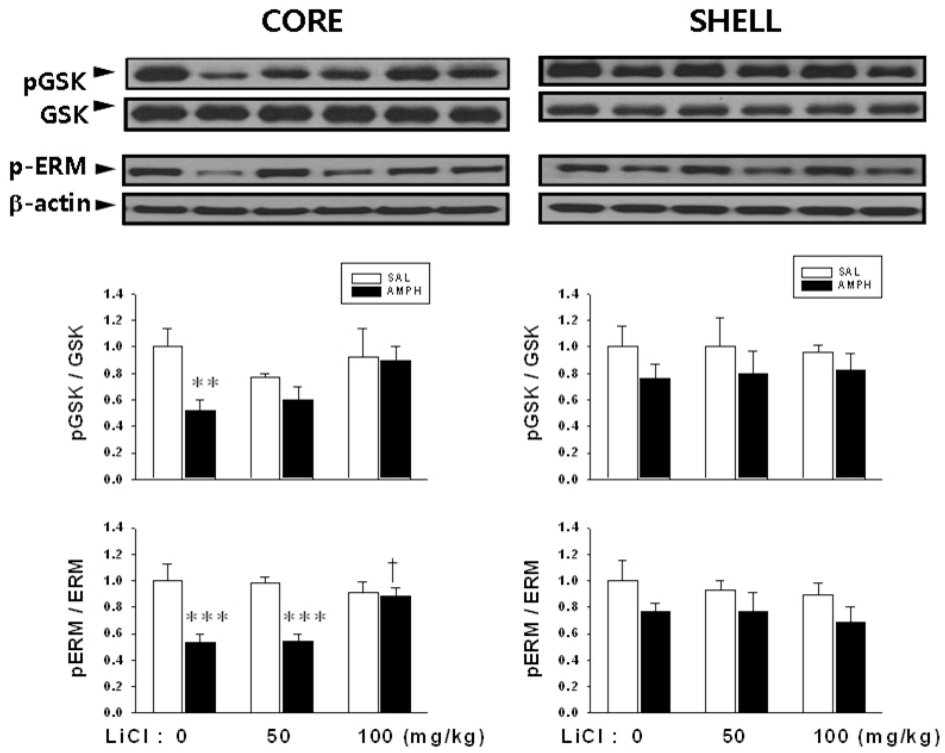


Figure 4. The recovery of the decreased phosphorylation levels of GSK and ERM proteins by lithium. Representative Western blots were shown. Values for the band intensities normalized to β-actin were expressed as mean ＋s.e.m. relative to vehicle control. Symbols indicate significant differences as revealed by *post-hoc* Bonferroni comparisons following two-way ANOVA. ** $p < 0.01$, *** $p < 0.001$, significantly different from vehicle-sal; † $p < 0.05$; significantly different from vehicle-AMPH (n=5 to 7 for GSK and 4 for ERM per group, respectively).

4. Microinjection of GSK activating peptide into the NAcc core decreases both pGSK and pERM levels in this site

In order to verify whether GSK signaling as upstream effectors regulates pERM levels in the NAcc core, an artificially synthesized small peptide (named as S9) which has similar amino acid sequences for phosphorylation site of GSK was microinjected into this site and both pGSK and pERM levels were measured. Interestingly, S9 dose-dependently decreased both pGSK and pERM levels in this site (ANOVA, $F_{2,11} = 4.32$; $p < 0.05$ and $F_{2,11} = 5.87$; $p < 0.05$, for pGSK and pERM, respectively) (Fig. 5). These results further suggest that AMPH-induced decrease of phosphorylated ERM levels in the NAcc core is under the regulation of GSK signaling.

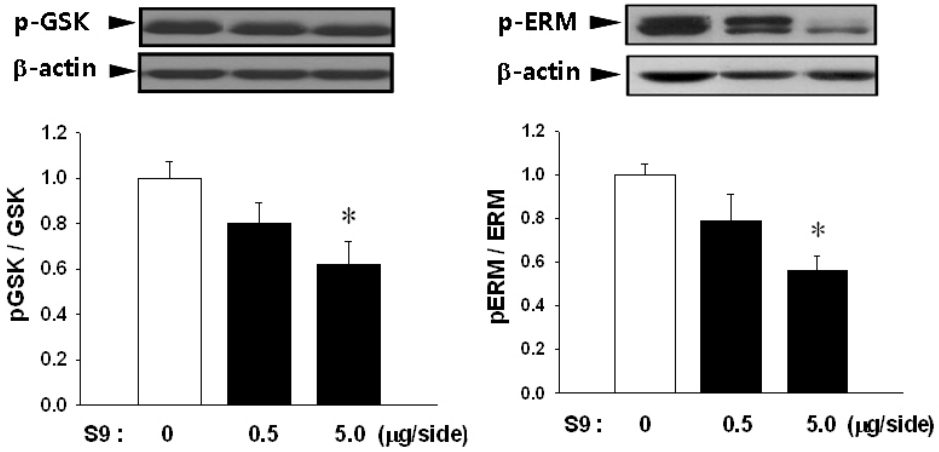


Figure 5. The decrease of both pGSK and pERM levels by S9 peptide, a GSK activator, in the NAcc core. The phosphorylation levels of both GSK and ERM in the NAcc core dose-dependently decreased by microinjection of S9 peptide into this site. Representative Western blots were shown. Values for the band intensities normalized to β-actin were expressed as mean + s.e.m. relative vehicle control (n=4 to 5 per group).

5. Microinjection into the NAcc core of PI3 kinase inhibitor, LY294002, decreases, while its activator, bpV, increases pERM levels in this site.

As shown in Fig. 3, AMPH decreases pAkt levels in the NAcc core, suggesting that PI3 kinase, which is upstream to GSK signaling, may involve in the regulation of pERM levels. In order to determine whether it is the case, both inhibitor (LY294002) and activator (bpV) for PI3 kinase are separately microinjected into the NAcc core and pERM levels were measured. As shown in Fig. 6, LY294002 decreased pERM levels in the NAcc core (ANOVA, $F_{2,19} = 22.50$; $p < 0.001$), while bpV dose-dependently increased pERM levels in this site (ANOVA, $F_{2,14} = 4.59$; $p < 0.05$). These results further suggest that AMPH-induced decrease of pERM levels in the NAcc core is also under the regulation of PI3 kinase signaling.

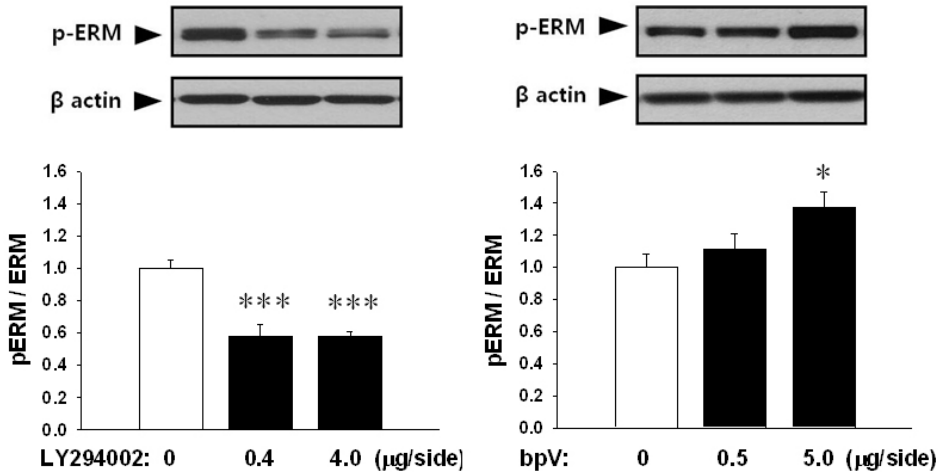


Figure 6. The change of pERM levels by microinjection into the NAcc core of PI3 kinase inhibitor, LY294002, or its activator, bpV. The phosphorylation levels of ERM in the NAcc core decreased by microinjection of LY294002 into this site. On the contrary, these effects were appeared to the opposite direction by microinjection of bpV into this site. Representative Western blots were shown. Values for the band intensities normalized to β-actin were expressed as mean + s.e.m. relative vehicle control (n=7 to 8 per group for LY294002 and 4 to 6 for bpV).

6. AMPH pre-exposure produces locomotor sensitization when challenged with AMPH after 2 weeks of withdrawal.

It is well-known that psychomotor stimulants like AMPH produces behavioral sensitization when repeatedly exposed to rats (Kalivas and Stewart, 1991; Koob and Le Moal, 2001). In order to examine whether AMPH pre-exposure may further regulate ERM proteins in the NAcc, rats were AMPH challenged after its pre-exposure and 2 weeks of drug-free withdrawal period. As expected, their locomotor activities were enhanced in AMPH compared to saline pre-exposed rats (Fig. 7). Interestingly, when measured the ratios of the phosphorylated to total levels for Akt, GSK, and ERM proteins, they were all decreased only in the core in the group of AMPH pre-exposed and saline challenged compared with the group of saline pre-exposed and saline challenged (Fig. 8). Two-way between-within ANOVA with pre-exposure as between and challenge as the within factors revealed significant effects of pre-exposure for both GSK and ERM ($F_{1,35} = 9.43$; $p < 0.01$, and $F_{1,35} = 8.41$; $p < 0.01$, respectively) in the core. It also revealed significant effect of pre-exposure x challenge interactions ($F_{1,35} = 4.19$; $p < 0.05$) for Akt proteins in the core. Interestingly, the decrease of pGSK levels was detected in the shell after challenge injection following AMPH pre-exposure and withdrawal period (Fig. 8B), suggesting that the shell is under different regulation from the core in terms of PI3 kinase-Akt-GSK signaling pathways. All together, these results indicate that chronic AMPH similar to acute injection regulates both Akt and GSK signaling in the same direction with ERM proteins in the NAcc core.

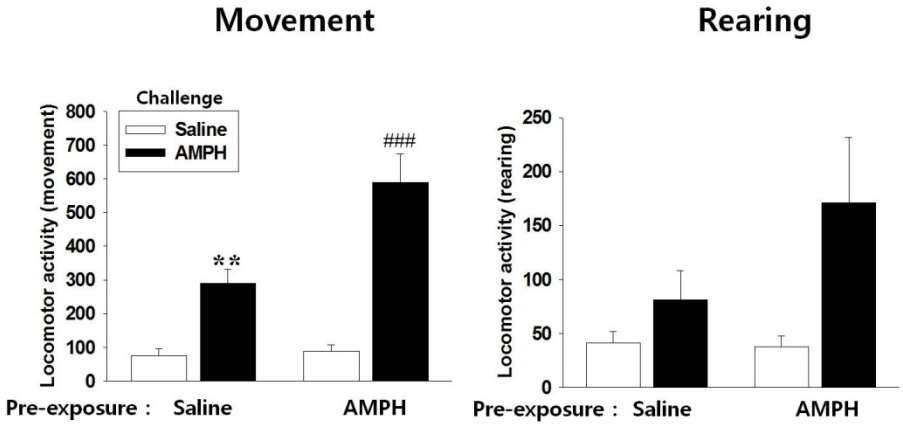
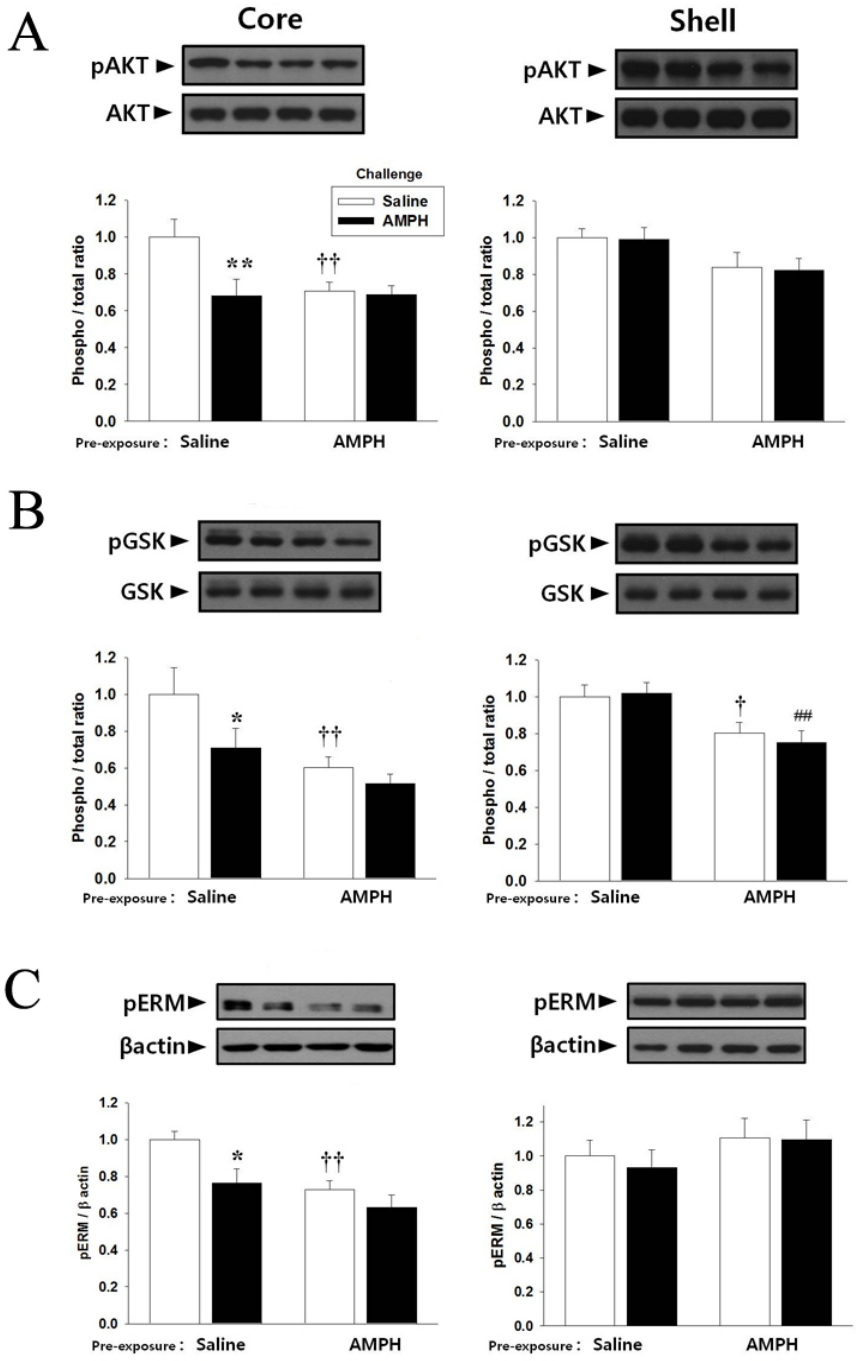


Figure 7. Locomotor sensitization produced by AMPH pre-exposure. Rats were pre-exposed with either saline or AMPH (1 mg/kg, IP) and they were challenged with the same dose of AMPH after 2 weeks of drug-free withdrawal. When measured for locomotor activity (both horizontal movement and rearing) for 1 hr after challenge of drug injection, AMPH compared to saline pre-exposed group showed significantly increased horizontal movement. Values for 1 hr total locomotor activity were expressed as mean + s.e.m. and symbols indicate significant differences as revealed by *post-hoc* Student-Newman-Keuls comparisons following two-way ANOVA. ** $p < 0.01$, significantly different from the group (saline pre-exposed and saline challenged); ### $p < 0.001$, significantly different from the group (saline pre-exposed and AMPH challenged) (n=9 to 10 per group).


A
Core
Shell
pAKT ►
AKT►
Challenge
Saline
AMPH
Phospho / total ratio
1.2
1.0
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0.4
0.2
0.0
**
††
Pre-exposure : Saline
AMPH
B
pGSK►
GSK►
*
††
†
##
C
pERM►
βactin►
pERM / β actin

Figure 8. The decrease of the basal levels of pAkt, pGSK and pERM by AMPH pre-exposure in the NAcc core. 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* Student-Newman-Keuls comparisons following two-way ANOVA. * $p < 0.05$, ** $p < 0.01$; † $p < 0.05$, †† $p < 0.01$; significantly different from the group (saline pre-exposed and saline challenged); ## $p < 0.01$, significantly different from the group (saline pre-exposed and AMPH challenged) (n=9 to 10 per group).

IV. DISCUSSION

In the present thesis, it has been first time showed that AMPH, even a single injection, can regulate the cellular status of structurally important ERM proteins by influencing PI3 kinase-Akt-GSK3β signaling in the NAcc core, but not in the shell. Acute AMPH decreased levels of pERM, pAKT and pGSK3β all in the same direction in the NAcc core. In addition, AMPH-induced decrease of pGSK3β and pERM levels were both significantly recovered back close to basal level by lithium, a GSK3β inhibitor, only in the NAcc core.

Further, interestingly, a direct bilateral microinjection into the NAcc core of S9 peptide, bpV or LY294002 all regulates ERM phosphorylation levels in the NAcc core. S9 peptide has similar amino acid sequences for phosphorylation site of GSK. Therefore, S9 peptide competes with GSK to block it's phosphorylation and plays a role as GSK activator. When S9 was microinjected into the NAcc core, it dose-dependently decreased both pGSK and pERM levels in this site. When microinjected, LY294002, a PI3 kinase inhibitor, decreased pERM levels in the NAcc core whereas bpV, a PI3 kinase activator, increased pERM levels in this site. These results further suggest that AMPH-induced decrease of phosphorylated ERM levels in the NAcc core is under the regulation of GSK and PI3 kinase signaling. Considering the role of ERM proteins in the cell-shape determination by crosslinking F-actin to plasma membrane,[9,10] these results suggest that even acute injection of AMPH is able to initiate possible cellular events leading to actin cytoskeletal remodeling by regulation of PI3

kinase-Akt-GSK3β signaling in the NAcc core (Fig. 9).

Interestingly, it has recently shown that the NAcc dendritic spine density is actually increased 6 hours after acute cocaine, another psychomotor stimulant similar to AMPH.[7] Thus, 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 considering, for example, the fact that the increase of dopamine levels by acute AMPH develops later to the sensitized increase in response to the same dose of drug when it is repeatedly administered.[43,44] And in the NAcc core cocaine treatment increased spine density only in the psychomotor sensitized group.[4] Actually, in the present results, it has been shown in the NAcc core, but not in the shell, that chronic AMPH-induced behavioral sensitization lowers the basal levels of pERM as well as pAkt and pGSK proteins in the same direction, supporting the idea that repeatedly exposed AMPH may further down-regulate pERM levels through PI3 kinase-Akt-GSK signaling pathways contributing to produce a more endurable biochemical changes leading to addicted behaviors.

The NAcc is a neuronal substrate that mediates motivated behaviors.[45] It is composed of two subregions, the core and shell, which are distinct structures with differential expression of neuropeptides, morphology, and synaptic inputs from different afferent structures.[45-49] On the basis of these anatomical profiles, the NAcc is functionally distinguished so that the core is thought to play a specific goal based behaviors on learning, whereas the shell seems to be crucial for unconditioned reward-seeking behaviors.[50] Several studies have

examined the differential roles of the NAcc core and shell in motivated behavior and the actions of drugs of abuse.[51] One study showed that rats with chronic administration of AMPH or cocaine exhibited sensitization of dopamine transmission in the NAcc core, but not in the shell.[52] Recently it was reported that AMPH sensitization is accompanied by increased c-*fos* mRNA expression in the NAcc core, while no effect of sensitization was seen in the NAcc shell.[47] In my own experiments, repeated intermittent exposure to AMPH produces behavioral sensitization that is associated with NAcc core. And it is well known that the development of behavioral sensitization by psychomotor stimulant drugs such as cocaine or AMPH consists of two phases; induction and expression, in which distinct neuronal substrates mediate different neuronal processes in the brain.[53,54] Evidence indicates that the development of behavioral sensitization is initiated in the VTA, while its expression is due to drug actions in the NAcc.[53] In the present experiment, the levels of pERM, pAKT, and pGSK are decreased not only AMPH sensitization group but also AMPH pre-exposed group without challenge in the NAcc core (not in the shell). These results suggest that the decreased levels of pERM, pAKT, and pGSK have important roles in the expression of behavioral sensitization after its development. However, it remains to be investigated in the future whether they are essential for induction of behavioral sensitization or not.

The present results indicate that AMPH differentially regulates pERM levels in these two subregions of the NAcc, suggesting that these effects may also differentially contribute to the specified role of the core and the shell. Interestingly, the decrease of pGSK levels was

detected in the shell after challenge injection following AMPH pre-exposure and withdrawal period (Fig. 8B). These results suggest that the shell is under different regulation from the core in terms of PI3 kinase-Akt-GSK signaling pathways.

Although detailed biochemical process needs to be examined (for example, as described in Ref. 1 and 55), the present results make it possible to propose that acute AMPH can induce a form of metaplasticity that alters synaptic ability to respond later in amplification, when animals are repeatedly exposed to this drug again, resulting in some morphologically better detectable figures as shown in animal models of drug addiction such as behavioral sensitization and self-administration.[4,5,56,57]

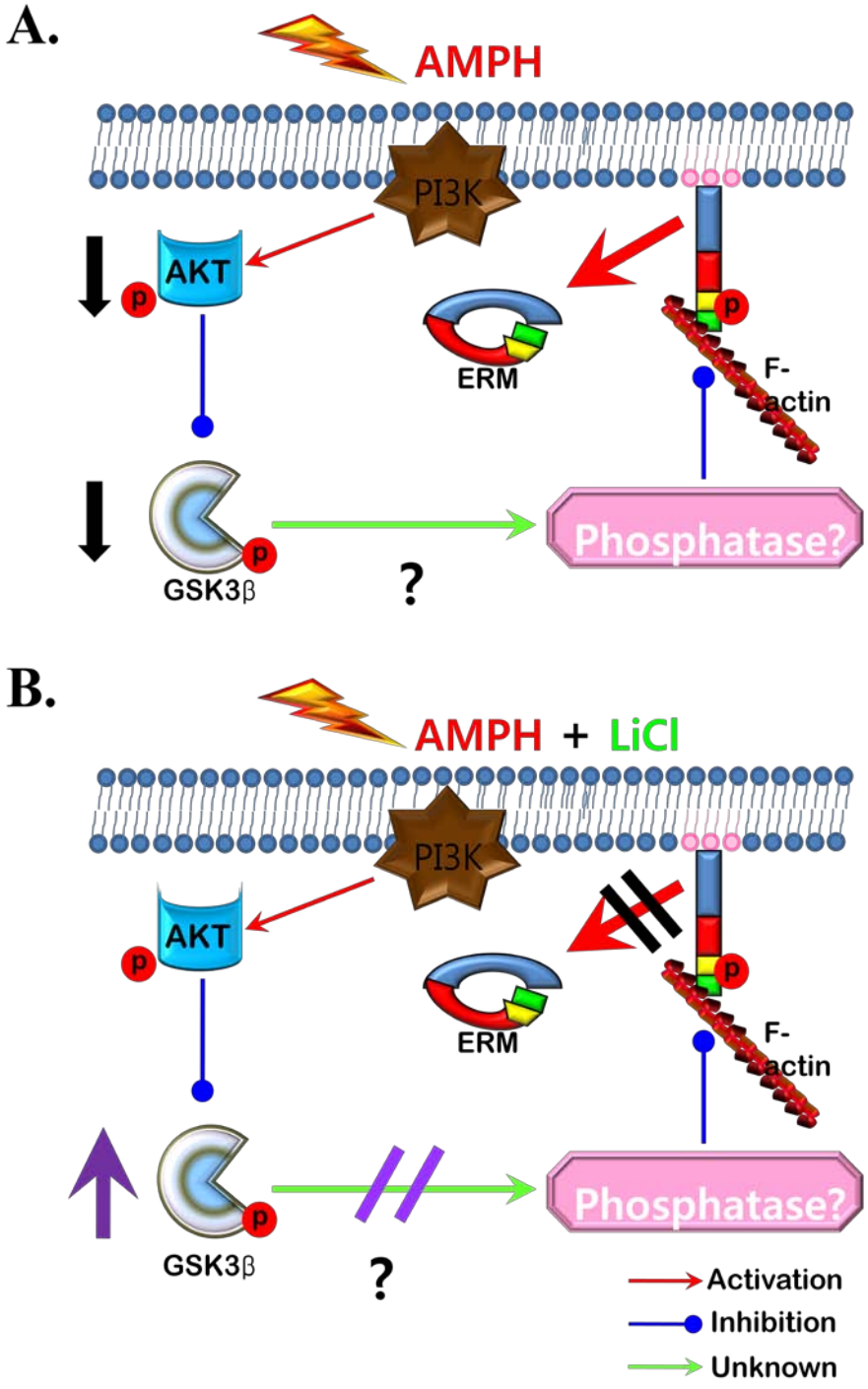
A.
AMPH
PI3K
AKT
p
ERM
p
F-
actin
p
GSK3β
?
Phosphatase?
B.
AMPH + LiCl
PI3K
AKT
p
ERM
p
F-
actin
p
GSK3β
?
Phosphatase?
Activation
Inhibition
Unknown

Figure. 9. A signaling pathway model that shows ERM signaling via PI3 kinase-Akt-GSK3β in acute AMPH in the NAcc core. A. AMPH decreases both pAkt and pGSK levels, leading to the decrease of pERM levels as well in the NAcc core. B. LiCl, when co-injected with AMPH, however, recovers pERM levels possibly via increase of pGSK levels.

V. CONCLUSION

1. Acute injection of AMPH decreases pERM levels in the same direction with pAkt and pGSK in the NAcc core, but not in the shell.

2. These results suggest that even acute injection of AMPH is able to initiate possible cellular events leading to actin cytoskeletal remodeling by regulation of PI3K-Akt-GSK3β signaling in the NAcc core.

3. Chronic AMPH-induced behavioral sensitization also lowers the basal levels of pERM as well as pAkt and pGSK proteins all in the same direction only in the NAcc core.

4. These results suggest that repeatedly exposed AMPH may down-regulate pERM levels through PI3 kinase-Akt-GSK signaling pathways in a longer-lasting fashion contributing to produce a more endurable biochemical changes leading to addicted behaviors.

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ABSTRACT(IN KOREAN)

중격측좌핵 심부에서 암페타민에 의한 ezrin-radixin-moesin 단백질의 glycogen-synthase-kinase 3β를 통한 신호전달 조절기전에 대한 연구

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장 주 경

중독성 약물인 암페타민과 코카인과 같은 정신자극제는중격측좌핵 내에서 도파민의 양을 증가시키고, 행동과민반응을 유도한다. 또한, 이때 중격측좌핵에서 수상돌기 가시의 수가 많아지는 것이 관찰되었음이 보고 된 바가 있다. Ezrin-radixin-moesin (ERM) 단백질은 서로 높은 상동성을 가지고 있으며, 구조 단백질인 섬유성 액틴 단백질을 원형질막과 연결 시켜주는 역할을 하면서 동시에 신호전달과정에

도 관여한다. 그러나 아직 ERM 단백질이 암페타민에 의해 조절 되는지에 대하여는 연구되지 않았다. 이를 바탕으로 본 연구에서는 중격측좌핵에서 ERM 단백질의 인산화가 암페타민에 의해 어떻게 조절을 받는가에 대해 알아보고자 하였다.

우선, 암페타민을 (2 mg/kg, IP) 한번 복강주입 하였을 때, 주입 후 15분부터 60분 까지 시간 의존적으로 중격측좌핵 심부에서만 ERM 단백질의 인산화가 현저하게 감소하였다. 또한 Akt와 GSK3β의 인산화도 ERM 단백질과 유사하게 시간 의존적으로 감소 하였다. 중격측좌핵 심부에서의 암페타민에 의한 ERM, Akt 그리고 GSK3β변화가 GSK3β에 의한 것인지 알아보기 위해서 암페타민과 GSK3β의 대표적 억제제인 리튬을 (100 mg/kg, IP) 쥐에게 주입하여보았다. 그 결과 중격측좌핵 심부에서만 암페타민에 의해 감소되었던 ERM이 GSK3β와 동일한 방향으로 회복됨을 볼 수 있었다. 더 나아가 GSK의 활성제인 S9 peptide (0.5 또는 5.0 ㎍/㎕)를 중격측좌핵 심부에 직접 주입함으로써 GSK3 β 와 ERM 단백질의 인산화가 감소되는 것을 확인하였고, 이번엔 GSK3의 상위 단계인 PI3 kinase의 억제제인 LY294002 (0.4 또는 4.0 ㎍/㎕) 또는 PI3 kinase의 활성제인 bpV (0.5 또는 5.0 ㎍/㎕)를 중격측좌핵 심부에 직접 주입함으로써 GSK3 β 와 ERM 단백질이 같은 방향으로 각각 감소하거나 증가함을 볼 수 있었다. 이 결과들을 통해서 암페타민이 중격측좌핵 심부에서 GSK3β를 통해 ERM 단백질을 감소시키는 것으로 사료된다.

암페타민을 (1 mg/kg, IP) 반복적으로 준 뒤, 약물이 없는 기간을 거친 후 동일한 약물을 다시 주게 되면, 약물에 대한 행동 반응이 증가하는 소위 행동과민반응이 나타남이

잘 알려져 있다. 이번 결과에서 반복적인 암페타민에 의한 행동과민 모델에서 중격측좌핵에서 어떤 변화가 있는지 관찰 하였는데 중격측좌핵 심부에서만 pERM 단백질이 pAkt와 pGSK와 같이 기저 수준이 감소함을 볼 수 있었다. 이는 반복적인 암페타민이 PI3 kinase-Akt-GSK 신호전달 기전을 통해 중독성 행동을 이끄는 좀 더 영속적인 생화학적 변화들을 생성할 수 있게 지속적이고 긴 방식으로 pERM 단백질을 낮게 조절하는 것으로 사료된다.

핵심되는 말 : 암페타민, ERM 단백질, Akt, GSK3β, 중격측좌핵 심부, 행동과민반응, 리튬, S9 peptide, bpV, LY294002

PUBLICATION LIST

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