Radixin in the nucleus accumbens contributes to the development of conditioned hyperactivity in an amphetamine-associated environment

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Department of Medical Science

The Graduate School, Yonsei University

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Directed by Professor Jeong-Hoon Kim

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2013년 1월 연구실 연구원으로부터 출발하여 대학원 입학 및 박사학위를 마치는 오늘 날까지 연구실에 몸담았던 지난 6년은 저에게 있어서 비단 심오한 신경과학과 약물중독에 대한 학문을 닦고 그 깊이와 전문성을 연마하는 시간이었을 뿐만 아니라 교수님과 연구실 구성원들과 함께 삶의 방방곳곳에서 수많은 기쁨과 재미를 창출했던 시간이었습니다. 그 시간들 속에서 의미들을 짚어가며 저 자신 또한 치유되고 감성과 지성이 모두 성장 할 수 있었습니다.

우선 먼저 좋은 학습환경과 연구터전을 만들어주시고 그 안에서 마음껏 상상하고 실천할 수 있게 아낌없는 지원과 격려를 해주신 김정훈 지도교수님께 감사의 마음을 전합니다. 연구분야에 첫 입문하여 깊은 바다와도 같았던 새로운 전문지식들을 습득하면서 때론 관문에 막히고 이해가 어려울 때 교수님으로부터 들었던 설명과 해석은 뇌리에 문제해결의 단서를 심어주었고 엉켜있었던 실마리들을 풀어주었습니다. 연구실 회의와 같은 공식적인 시간뿐만 아니라 일상 중 교수님과 함께 식사를 하고 차를 마시면서 소통했던 시간들 속에서도 공부의 유익함과 즐거움

을 새감 깨닫게 되었고 동기부여가 되었습니다. 실험의욕이 앞섰던 시절 실수도 있었지만 문제에 대한 정확한 조명과 함께 또 너그럽게 받아주시고 응원해주신 마음 잊지 못합니다. 수많은 실험데이터 속에서 번뇌하면서 의미를 찾아가며 함께 쾌재를 불렀던 순간은 의과학자로 성장하는 저에게 큰 기쁨과 보상이 되었고 학문과 연구에 더 큰 매력을 느끼게 하였습니다.

그 밖에도 연구실의 활력소이고 아이디어 뱅크이며 유학생활을 하는 저에게 따뜻한 삶의 온정을 느끼도록 물심양면으로 도와주신 선생님이지만 누나와도 같은 김화영 박사님께 고마운 마음을 전합니다. 그리고 언제나 성심성의것 실험결과에 대하여 충분히 토론해주시는 윤형신 박사님, 지금은 졸업하여 미국으로 새로운 도전을 향해 나섰지만 그전까지도 박사학위논문 작성관련 소중한 조언을 해주던 조보람 박사님, 그때 그 시절 아이스크림을 먹고 함께 신촌 캠퍼스를 거닐었던 이정원 박사님, 연구실생활 초반부터 저에게 많은 도움을 주었고 작은 소식에도 크게 축하해주는 배려와 리액션의 아이콘 곽명지 석사님, 어려운 난제도 침착하게 풀어나가는 연구실 새로운 <공대언니> 구민정 석사님, 이젠 연구실의 핵심인력으로 성장 및 도약해 나가는 씩씩한 사나이 한준엽 학사님, 아직도 숨

겨진 재능이 더 많은 이서현 학사님 그리고 연구실의 앞날을 짊어질 새 얼굴 박종우, 임하은 학사님들에게도 감사의 마음을 전합니다. 졸업하였어도 늘 연구실의 후배들에게 관심을 가져주시고 달려와 주시는 김승우 박사님, 장주경 박사님, 신중근 석사님에게도 감사의 마음을 표합니다. 그리고 인생의 교차점에 서있었던 저에게 연세대학교 대학원에서 박사학위를 할 수 있도록 기도해 주시고 응원해 주신 박순복 교수님, 듀크대학의 유재성 교수님께도 감사의 마음을 전합니다.

연구계획 자문심사부터 본 심사에 이르기까지 귀한 시간을 내어 지도 편달하여 주시고 논문이 더욱 빛 날수 있도록 아낌없는 조언을 하여 주신 이종은 교수님, 정영철 교수님, 정호성 교수님께도 깊이 감사를 드립니다.

마지막으로, 무엇보다도 학위과정 동안 저를 믿어주고 인내해주고 지지해준 아내와 학위과정 중 태어난 딸을 정성껏 보살펴주신 장모님, 장인어른과 늦깎이 박사가 되기까지 헌신해주신 부모님께 이 논문을 바칩니다.

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ABSTRACT

Radixin in the nucleus accumbens contributes to the development of conditioned hyperactivity in an amphetamine-associated environment

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(Directed by professor Jeong-Hoon Kim)

The nucleus accumbens (NAcc) is an important neuronal substrate mediating rewarding effects of drugs of abuse in the brain. It has been suggested that repeated exposure to psychostimulants induced dendritic spine plasticity in the NAcc, which is thought to be related with enduring drug-related behaviors including conditioning (associative form of memory) and behavioral sensitization (non-associative form of memory). Dendritic spines are postsynaptic structures located on dendritic shafts of neurons where they mediate electrical neurotransmission responding to excitatory axon terminals and can be classified into thin, stubby and mushroom based on their different size and shapes. Structural plasticity of dendritic spines has its basis on

reorganization of actin cytoskeleton which is controlled by various actin binding proteins including ezrin-radixin-moesin (ERM) proteins. Previously, it has been shown that ERM proteins are expressed in the brain, most abundantly in neurogenic regions. However, there are few studies directly showing their presence and subcellular distribution on adult neurons in vivo. In my thesis, first I aimed to examine the subcellular distribution of radixin, one of ERM proteins, in the NAcc. In addition, ERM proteins have been implicated in cell-shape determination by crosslinking F-actin to plasma membrane. Thus, it was hypothesized that there might be a strong link between ERM proteins and dendritic spine plasticity in response to drugs of abuse. So, next, I aimed to investigate whether there are any effects of accumbal radixin in amphetamine-induced conditioning and sensitization. The lentiviral constructs expressing copGFP alone (GFP), radixin wild-type (Rdx-WT) or a pseudo-active radixin mutant T564D (Rdx-MUT) were injected into the NAcc of rats. It was found that Rdx-WT overexpression prevented the expression of conditioned locomotion in paired rats when compared to those infected with either GFP or Rdx-MUT in the same group. Moreover, Rdx-WT overexpression in the NAcc also prevented the expression of amphetamine-induced hyper-locomotion in a subsequent sensitization test in paired rats, while no behavioral changes were observed in paired rats overexpressing either GFP only or Rdx-MUT. Interestingly, the inhibition of the expression of sensitized locomotor responses shown in Unpaired GFP and MUT rats, a phenomenon termed as conditioned inhibition, was saved when Rdx-WT was overexpressed in the NAcc.

Finally, it was found that the behavioral effect of Rdx-WT in paired rats was correlated with the number of total spine density in the NAcc. Among the sub-types of spines, thin spines are found to be the major contributor to this effect. These data, first time to my knowledge, demonstrated that radixin in the NAcc specifically contributes to amphetamine-induced associated memory (i.e., conditioning and

context-specific sensitization) possibly through structural plasticity of dendritic spines in the NAcc.

Key words: Radixin, nucleus accumbens, conditioned activity, dendritic spine, amphetamine

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I. INTRODUCTION

Drug addiction can be defined as compulsive drug seeking and use, despite of harmful consequences.[1] One common feature of drug addiction in clinic is pathological desire or urge to drugs of abuse,[2] which increases risk for relapsing in addicts,[3,4] and has been proposed as a result of the disturbances of brain reward systems.[5] In order to elucidate the mechanism underling the neuroadaptations that maintain pathological drug seeking behavior, animal models are frequently used. For example, rats repeatedly exposed to psychomotor stimulants such as cocaine and amphetamine (AMPH) produce a phenomenon, called behavioral sensitization, which is manifested as enhanced increase of locomotor activity when subsequently challenged with the drug after a withdrawal period.[6] Once behavioral sensitization

develops, it is maintained for a long period of time as a form of long-term memory.[6] Thus, behavioral sensitization has been proposed as a model reflecting a certain aspect of human addiction, since it shares such characteristics as escalation of drug use and long-lasting craving.[6-8]

Behavioral sensitization induced by psychomotor stimulants often occurs as the result of alterations in the overflow of neurotransmitters in brain reward systems.[9,10] It has been reported that behavioral sensitization accompanied the augmented dopamine (DA) and glutamate overflow in the nucleus accumbens (NAcc).[6,11] The NAcc receives dopaminergic projections from ventral tegmental area in the midbrain and glutamatergic projections from prefrontal cortex, hippocampus and amygdala.[12] These neuroanatomical connectivity posits the NAcc as a center of brain reward system, which mediates locomotor activating and rewarding effects of drugs of abuse.[13]

Interestingly, the behavioral sensitization produced by repeated exposure of psychomotor stimulants is further modulated by environmental context.[14] Drugs of abuse often confer their locomotor activating and rewarding properties to the environments in which they were given, resulting in the development of long-lasting conditioned effects to the environment itself even in the absence of drugs.[15] In other words, with repeated drug use, a neutral stimulus in the environment where the drugs are taken can gradually acquire rewarding effects of drugs of abuse by forming a new associative memory between drugs and environment in the brain.[7,16-18] Such Pavlovian conditioning process has been suggested to additionally contribute to craving and consequently promote relapse in addicts.[19,20] Though behavioral sensitization, as a long-lasting non-associative form of memory induced by drugs of abuse, has its own mechanism separated and distinguished from the development of conditioning, its expression can come under the control of a specific environmental

context.[15] For example, evidence from discriminative conditioning procedure showed that sensitized locomotion can only be observed in animals previously exposed to the drug in test environment (paired), while the contexts in which the drug is not expected (unpaired) prevented the expression of behavioral sensitization. This phenomenon has been called as context-specific sensitization.[14] These findings suggest that, in a specific experimental setting, excitatory conditioning can enhance sensitized response in paired animals, while conditioned inhibitory mechanisms may inhibit the expression of sensitization in unpaired animals.[14,18]

Dendritic spines are postsynaptic structures located on dendritic shafts of neurons and can be classified into thin, stubby and mushroom based on their different size and shapes.[21] In relation to learning and memory, it has been suggested that experience-dependent changes in behavior are associated with the alterations in the quantity and the structure of dendritic spines (i.e., structural plasticity) in relevant brain areas.[22] For example, it has been shown that associative memory formation not only increased the number of dendritic spines but also changed synaptic morphology in the hippocampus.[23,24] Evidence also indicates that structural plasticity of dendritic spines especially in the NAcc of brain reward circuitry has been implicated in drugs of abuse and thought to contribute to the development of drug addiction.[25-27]

Structural plasticity of dendritic spine requires reorganization of actin cytoskeleton which is controlled by various actin binding proteins.[28,29] Ezrin, radixin and moesin constitute ERM proteins (about 82, 80, 75kDa in molecular weight, respectively), and they are structurally characterized by containing the N-terminal FERM domain and C-terminal actin-binding domain in which they show high degree of amino acid identity.[30,31] Inactive ERM proteins structurally show a “closed” conformation, which masks the regulatory N- and C- terminal domains of ERM

proteins. When a threonine (Thr) residue at C-terminal actin-binding domain is phosphorylated, the "closed" conformation become opened up and activated by exposing the N-terminal FERM and C-terminal actin-binding domains.[32] Activated ERM proteins, which are recruited to the below of cell membrane surface and act as cross-linkers between the plasma membranes and cytoskeleton via their N- and C-terminal domains, have been implicated in various cellular processes such as microvilli formation, cell adhesion, cell motility, cytokinesis, and the integration of membrane transport with signaling pathways.[33]

Although there are some studies showing that structural plasticity of dendritic spines is associated with the development of addiction process, little is known about the role of ERM proteins in the NAcc in such process. Interestingly, it has been previously shown that the phosphorylation levels of ERM proteins in the NAcc are decreased by acute psychomotor stimulants like AMPH and cocaine,[34,35] suggesting that the activity of ERM proteins in the NAcc is under the regulation of drugs of abuse. Considering the linkage between ERM proteins and F-actin, it is possible that regulation of ERM proteins by psychomotor stimulants may induce actin cytoskeleton remodeling and subsequent morphological changes of dendritic spine in the NAcc.[34]

Although ERM proteins are abundantly expressed in the NAcc, there are few studies directly showing their presence and subcellular distribution on adult neurons in vivo. Thus, in the present thesis, it was examined: first, the subcellular distribution of radixin on the medium spiny neurons (MSNs) of rat NAcc by using immunofluorescence staining and confocal microscopy; second, how radixin in the NAcc affects the addictive behaviors both in behavioral sensitization and Pavlovian conditioning animal models by manipulating its expression levels in this site; third, the structural plasticity of dendritic spines accompanied with the behavioral changes.

II. MATERIALS AND METHODS

1. Animals and drugs

Male Sprague-Dawley rats weighing 200-230 g (equivalent to 6 weeks olds) on arrival were obtained from Orient Bio Inc. (Seongnam-si, Korea). They were housed three per cage in a 12-hours light/dark cycle room (lights out at 8:00 pm), and all experiments were conducted during the day time. Rats have 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 of Yonsei University College of Medicine.

D-amphetamine sulfate (United States Pharmacopeial, Rockville, MD, USA) was dissolved in sterile 0.9% saline to a final working concentration of 1 mg/ml.

2. Adeno-associated virus surgeries and infusions

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 skull surface was exposed. Infusion cannulas (28 gauge, Plastics One, Roanoke, VA, USA) connected to 2 μl Hamilton syringes (Reno, NV, USA) via PE-20 tubing were angled at 10° to the vertical and lowered into the NAcc core (A/P, +3.2; L, +2.8; D/V, –7.1 mm from bregma and skull). 1 μl (1×10^{11} GC/ml) of adeno-associated viral vectors (AAV) (Vector Biolabs, PA, USA), expressing enhanced Green Fluorescent Protein (eGFP) were then bilaterally infused for 1 minute and another 10 minutes allowed for diffusion before the infusion cannulas were taken out. After viral injection, rats were returned to their home cages for 2 weeks of recovery period.

3. Tissue preparation and immunohistochemistry of radixin

Rats were deeply anesthetized with intraperitoneal (IP) ketamine (100 mg/kg) and xylazine (6 mg/kg) and perfused with intracardially with ice-cold 1xphosphate buffered saline (PBS, pH 7.4). Thereafter, the brains were quickly removed and immediately cut into blocks containing NAcc on an ice cold dish. The tissue blocks were then plunged into ice-cold 4% paraformaldehyde (in 1X PBS, pH 7.4) and maintained for one hour. After rinsed with 1X PBS, tissue blocks were transferred to 30% sucrose and stored overnight at 4℃. Tissue blocks were embedded and stored at -80℃ on the next day.

For immunofluorescence staining, free-floating brain sections (40 μm) from frozen tissues were prepared on a freezing microtome (HM 525, Thermo Scientific, Waltham, MA, USA). They were blocked for 1 hour in 1X PBS containing 5% normal goat serum (Vector laboratories, Burlingame, CA, USA) and 0.3% triton X-100 and then incubated overnight rabbit monoclonal anti-radixin antibody (1:1000, Abcam, Cambridge, UK) diluted in 1X PBS containing 2% normal goat serum and 0.1% triton X-100 at 4℃. Following overnight incubation, sections were rinsed 3 times for 10 minutes in 1X PBS containing 0.1% triton X-100 and incubated for 2 hours with the secondary antibodies which were goat anti-rabbit Alexa Fluor 568 (1:1000, Thermo Scientific, Waltham, MA, USA) at room temperature in the same diluent as primary antibodies. After 2 hours, they were rinsed 3 times for 10 minutes again in 1X PBS containing 0.1% triton X-100 and cover-slipped with Vetashield mounting medium H1400 (Vector laboratories, Peterborough, UK).

For double immunofluorescence staining, tissue sections were incubated with mixture of primary antibodies following tissue blocking. The following primary antibodies were used: rabbit monoclonal anti-radixin antibody (1:1000, Abcam, Cambridge, UK), mouse monoclonal anti-PSD95 antibody (1:1000, Abcam,

Cambridge, UK), chicken polyclonal anti-GFP antibody (1:1000, Abcam, Cambridge, UK). The following secondary antibodies were used: goat anti-rabbit Alexa Fluor 568 (1:1000, Thermo Scientific, Waltham, MA, USA), goat anti-mouse Alexa Fluor 488 (1:1000, Thermo Scientific, Waltham, MA, USA), goat anti chicken Alexa Fluor 488 (1:1000, Thermo Scientific, Waltham, MA, USA).

For visualizing radixin immunoreactivity, immunofluorescence sections were examined under either epifluorescence microscopy (Olympus, Tokyo, Japan) or LSM700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) equipped with diode lasers at wavelength of 488 and 555 nm. All of the low-magnification images were taken under epifluorescence microscopy with the same exposure time. For high-magnification images, each NAcc region of image stacks spaced by 0.5 μm in z plane were acquired as 1024 x 1024 pixels per x-y frame with 1 Airy unit of optical thickness with 63x oil immersion objective (Numerical aperture 1.4). Scan averaging was set to 4 to improve signal to noise ratio of each optical section.

4. Generation of lentivirus containing radixin

Lentiviruses containing radixin (both wild-type and mutant) were obtained as a gift from Dr. Lars Riecken at the Fritz Lipman Institute in Germany. In brief, human wild-type radixin and radixin T564D were cloned in the lentiviral pCDH-CuO-MCS-EF1-copGFP vector (SystemBiosciences, Mountain View, CA, USA). To generate stable NIH3T3 clones, cells were transduced with lentiviral particles coding for copGFP and either Rdx-WT or RdxT564D. Viral particles were concentrated threefold by polyethylene glycol precipitation (PEG-6000; Sigma). Polybrene (Sigma) was added to a final concentration of 8 μg/ml. GFP-positive cells were selected by FACS.

5. Lentivirus surgeries and infusions

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 skull surface was exposed. Infusion cannulas (28 gauge, Plastics One, Roanoke, VA) connected to 2 μl Hamilton syringes (Reno, NV, USA) via PE-20 tubing were angled at 10° to the vertical and lowered into the NAcc core (A/P, +3.2; L, +2.8; D/V, –7.1 mm from bregma and skull). 1 μl (1 x 10^{12} particles/ml) of lentiviral constructs, either expressing copGFP alone (termed GFP), or copGFP with radixin wild-type (termed Rdx-WT), or copGFP with constitutive phosphomimetic radixin mutant T564D (termed Rdx-MUT), were then bilaterally infused for 1 minute and another 10 minutes allowed for diffusion before the infusion cannulas were taken out. After viral injection, rats were returned to their home cages for 2 weeks of recovery period.

6. Locomotor activity apparatus

Locomotor activity was measured with a bank of nine activity boxes (35 × 25 × 40 cm) (IWOO Scientific Corporation, Seoul, Korea) made of translucent Plexiglas. Each box was individually housed in a PVC plastic sound attenuating cubicle. The floor of each box consisted of 21 stainless steel rods (5 mm diameter) spaced 1.2 cm apart center-to-center. 2 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 the box, were used to estimate locomotor activity.

7. Experimental design and procedures

After 2 weeks of surgical recovery, rats with different viruses (GFP, Rdx-WT,

Rdx-MUT) were randomly assigned to 3 groups according to conditioning paradigm. During conditioning development, rats were administered IP injections of saline or AMPH in five 2-day blocks. For each block, rats received saline in their home cages on day 1 and amphetamine (1 mg/kg, IP) in locomotor activity boxes on day 2 (Paired), amphetamine in their home cages on day 1 and saline in the activity boxes on day 2 (Unpaired), or saline in both environments (Control). On the conditioning test, 1 week after the last conditioning block, all rats were tested for their conditioned locomotor response in the activity boxes for 1 hour following an IP saline injection. Additionally, sensitization test was conducted after 2 days of conditioning test. On the test day, they were all habituated in the locomotor activity boxes for 30 minutes and tested for their locomotor response in the activity boxes for 1 hour following an AMPH (1 mg/kg, IP) challenge (see Figure 1).

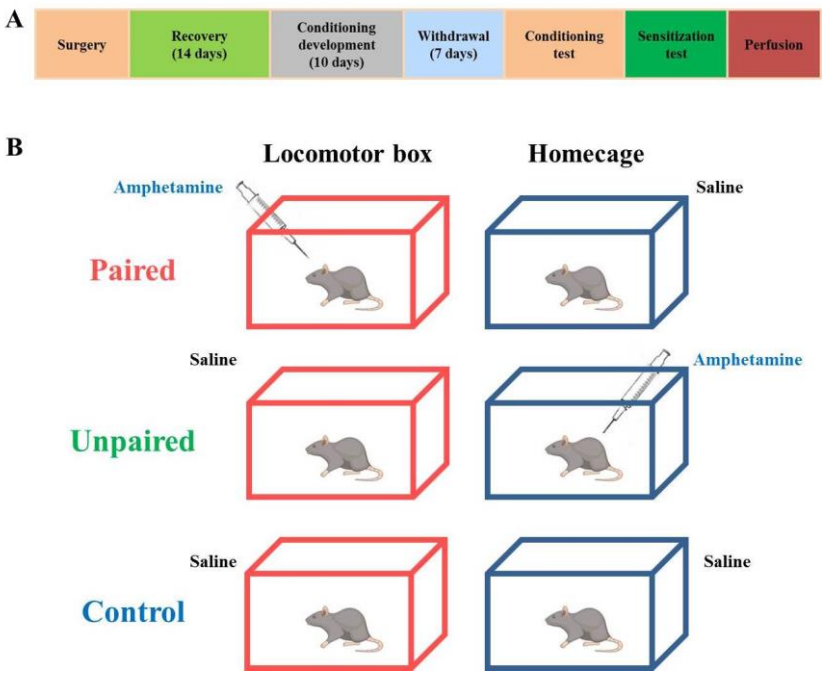


Figure 1. Experimental design and procedures. (A) Experimental timeline. (B) Model describing the conditioning paradigm.

8. Tissue preparation and immunohistochemistry for spine analysis

On the next day after amphetamine challenge, rats were all deeply anesthetized with ketamine (100 mg/kg) and xylazine (6 mg/kg) and then perfused transcardially with 1X PBS (pH 7.4) followed by 4% paraformaldehyde solution in 1X PBS (pH 7.4). Then, the brains were removed and transferred to ice-cold 4% paraformaldehyde in 1X PBS (pH 7.4) for another 24 hours. Then, blocks were washed with 1X PBS, cryoprotected in 30% sucrose solution and stored at -80℃.

100 μm free-floating sections from frozen tissue blocks were prepared on freezing microtome (HM 525, Thermo Scientific, Waltham, MA, USA). To acquire enhanced GFP signals from lentiviral vector infected neurons, GFP staining was conducted in tissue sections. This approach has been applied to increase the brightness of sub-micron dendritic spines in infected neurons.[36,37] 100 μm tissue sections were blocked for 1 hour in 1X PBS containing 5% normal goat serum (Jackson Immunoresearch Laboratories, West Grove, PA, USA) and 0.3% triton X-100. Then, they were incubated overnight with anti-TurboGFP (rabbit polycolonal, 1:1000, Evrogen, Moscow, Russia) antibodies diluted in 1X PBS containing 2% normal goat serum and 0.1% triton X-100 at 4℃. Following overnight incubation, the sections were rinsed 3 times in 1X PBS containing 0.1% triton X-100 and incubated with the anti-rabbit secondary antibody coupled to Alexa 488 (1:1000; Invitrogen, Waltham, MA, USA) for 2 hours at room temperature. They were rinsed again and coverslipped with Vetashield mounting medium H1400 (Vector laboratories, Peterborough, UK).

9. Spine imaging and analysis

For spine analysis, individual dendritic segments of medium spiny neurons

(MSNs) were imaged based on published requirements[38]: (1) the segments have to appear uniform GFP distribution, (2) the segments can not be overlapped with other neighboring dendritic segments and be traced back to their soma, (3) secondary and tertiary dendritic segments are chosen at least 50 μm away from soma. Terminal segments were excluded. 1 to 3 dendrites per neuron and three to seven neurons per rat were imaged. Spine analyses were conducted by experimenter who knew only tissue numbers, but was blind to what group they belong.

All images were acquired under a LSM700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) with a 488 nm argon laser. For whole cell reconstructions, confocal stacks of MSNs were imaged at 20x air objective lens (numerical aperture 0.7) with a z-step size of 0.99 μm and an XY resolution of 0.417 μm. For dendrite imaging, dendritic segments stacks spaced 0.2 μm were acquired with a 63x oil-immersion objective (numerical aperture 1.4) and a scan zoom of 3.0. The pinhole aperture set to 1 Airy Unit and the line average of 2 was used. The full dynamic ranges of images were obtained by adjusting the laser intensity and photomultiplier tube (PMT) gain. All images were taken with a resolution of 1024 pixels in x dimension and the y dimension within the frame was cropped to ~ 300 pixels according to particular dendritic segments for fast image acquisition, the pixel dwell time was 1.58 μm/s. The final voxel size was 0.033 x 0.033 x 0.2 μm^3 in x-y-z plane.

To improve contrast and resolution, raw confocal images were deconvolved with AutoQuant X3 deconvolution software (Media Cybernetics, Inc, Rockville, MD, USA). Dendrite tracing and automatic spine detection was then performed using Neuronstudio software with rayburst algorithm, which classifies spines into thin, mushroom or stubby according to the classification dimensions (i.e neck ratio, thin ratio, mushroom size).[39] The minimum and maximum spine height values were set at

0.5 μm and 3.0 μm. 22 voxels were set for minimum stubby size upon published criteria.[40]

10. Statistical analyses

Statistical analyses were performed using the Sigma Plot version 12.0 (Systat Software, San Jose, CA, USA). The data obtained from locomotor activity are expressed as the mean + SEM and analyzed either with 2-way analysis of variance (ANOVA) or 2-way repeated measures ANOVA, followed by Student-Newman-Keuls (SNK) *post hoc* test. The total, thin, stubby and mushroom spine densities per rat from each group are expressed as the mean + SEM and analyzed with 2-way ANOVA, followed by SNK *post* hoc test. Differences between experimental conditions were considered statistically significant when $p < 0.05$.

III. RESULTS

1. Subcellular distribution of radixin in the medium spiny neurons of rat NAcc

GABAergic MSNs constitute predominant cell type in the NAcc and play a major role in drug addiction.[41] MSNs undergo long-lasting morphological adaptations upon chronic psychomotor stimulants.[42] Previous studies have shown that ERM proteins are expressed in embryonic and adult brain.[43-45] However, nothing is known about the subcellular distribution of ERM in the MSNs of rat NAcc. Thus, in this thesis, it was focused on the radixin, one of the major ERM protein mostly expressed in the NAcc, and examined the subcellular distribution of this protein in the MSNs of rat NAcc using immunofluorescence staining and confocal microscopy.

Consistent with previous results,[45] radixin expression was successfully detected in the subventricular zone (SVZ) (Figure 2A). Confocal images at high magnification showed that high density of radixin immunoreactivity was evident in the NAcc (Figure 2C and D). Next, in order to investigate the subcellular localization of radixin in the NAcc, double immunofluorescence staining of radixin and PSD95, a marker of excitatory postsynaptic sites, was performed. For comparison, it was also performed co-immunostaining of PSD95 and GluR1 in this site, which was used as positive control of colocalization. Distinct labeling patterns were observed between radixin and PSD95, suggestive of no existence of radixin on postsynaptic spines (Figure 3A - C). By contrast, the image of PSD95 and GluR1 shows the massive colocalization in this site (Figure 3D). To directly confirm the subcellular localization of radixin in the MSNs, AAVs encoding eGFP were microinjected into the NAcc. After 2 weeks, brain sections from virus injected rats were processed for immunofluorescence staining for radixin, combined with GFP to

enhance the brightness of sub-micron dendritic spines in virus infected neurons (Figure 4A). Interestingly, radixin clusters were found to be localized within eGFP positive dendrites of MSNs. Moreover, orthogonal section mode showed that radixin clusters were in close apposition to membrane sides of dendrites (Figure 4B - E). However, the radixin clusters were not observed directly on dendritic spines in the MSNs.

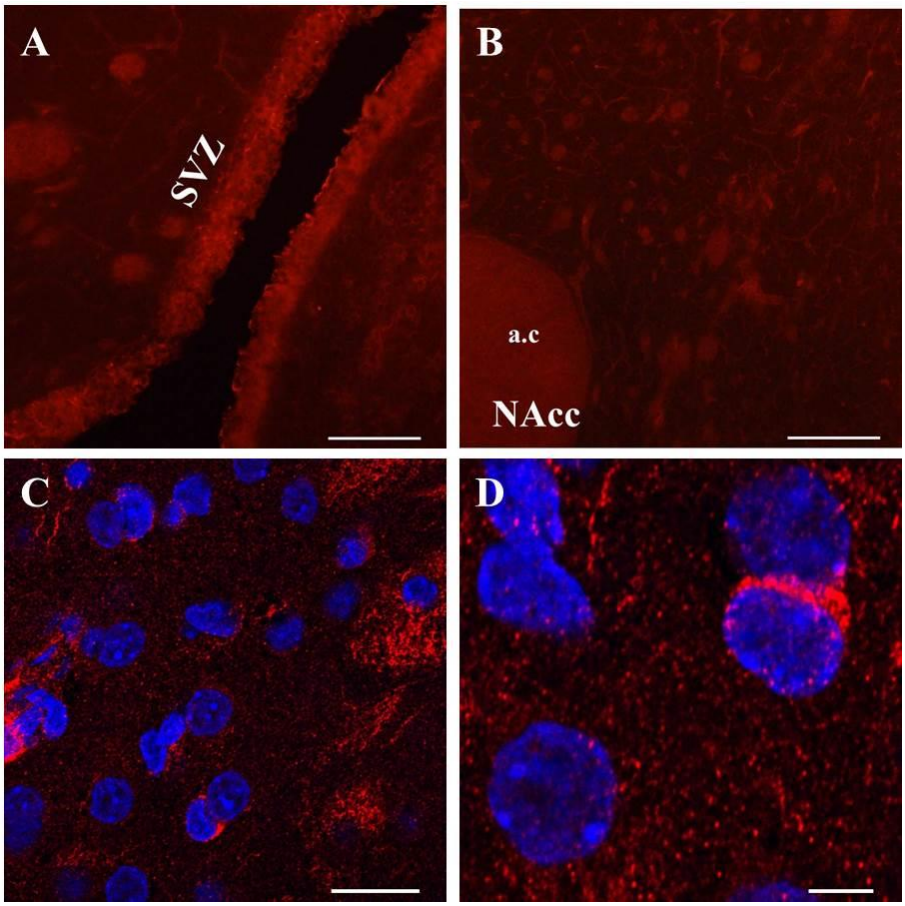


Figure 2. Immunofluorescence for radixin in the NAcc. (A, B) Images at low magnification from epifluorescence microscopy showed that intense expression of radixin (red) along the SVZ and in the NAcc of adult rat brain. (C) Images at high magnification from confocal microscopy revealed radixin immunoreactivity structures in the NAcc. 4,6-diamidino-2-phenylindo (DAPI) was used for nucleus staining (blue). (D) Especially, note the high density of radixin signals surrounding the GABAergic MSNs. SVZ, subventricular zone; NAcc, nucleus accumbens; a.c, anterior commissure. Scale bars=100 μm (A), 200 μm (B), 20 μm (C), 5 μm (D).

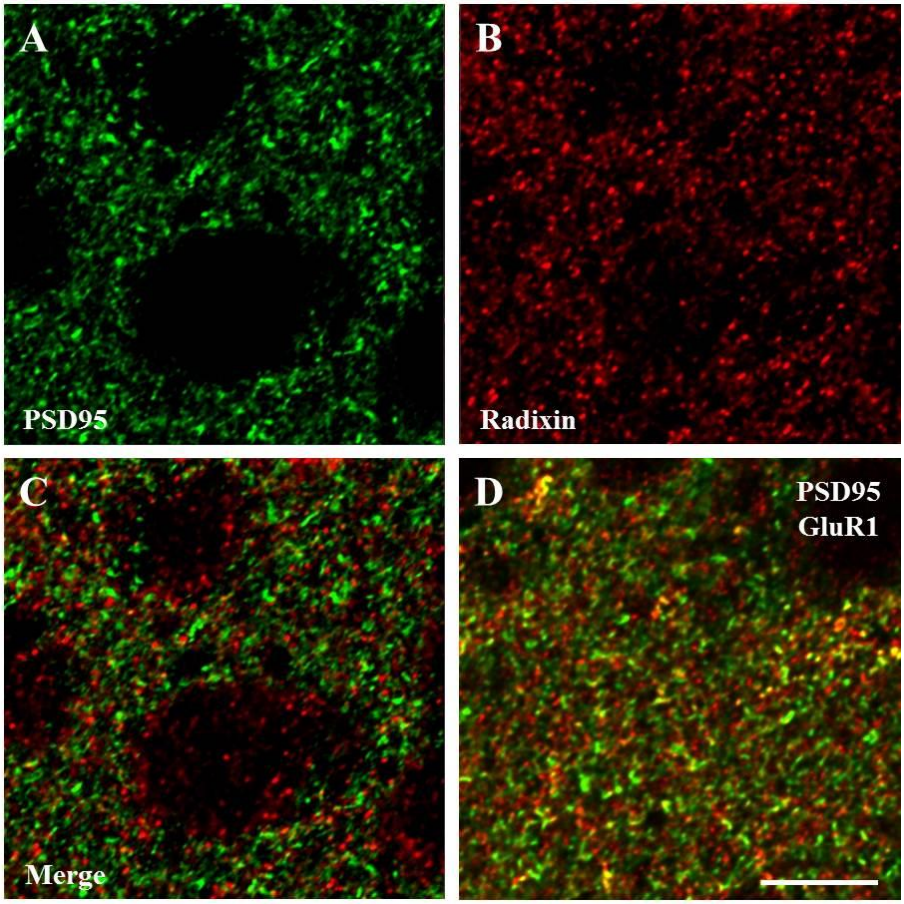


Figure 3. Immunofluorescence images of double staining of PSD95 and radixin in the NAcc. (A, B) Immunofluorescence staining for PSD95 (green) and radixin (red) in the NAcc. (C) Distinct labeling patterns were observed between PSD95 and radixin in the NAcc. Note that radixin-positive puncta are not colocalized with PSD95-positive puncta that represent excitatory postsynaptic sites. (D) Immunofluorescence staining of PSD95 (green) and GluR1 (red) in the NAcc shows extensive colocalization between two proteins, as confirmed by yellow puncta in the image. Scale bars=5 μm (D).

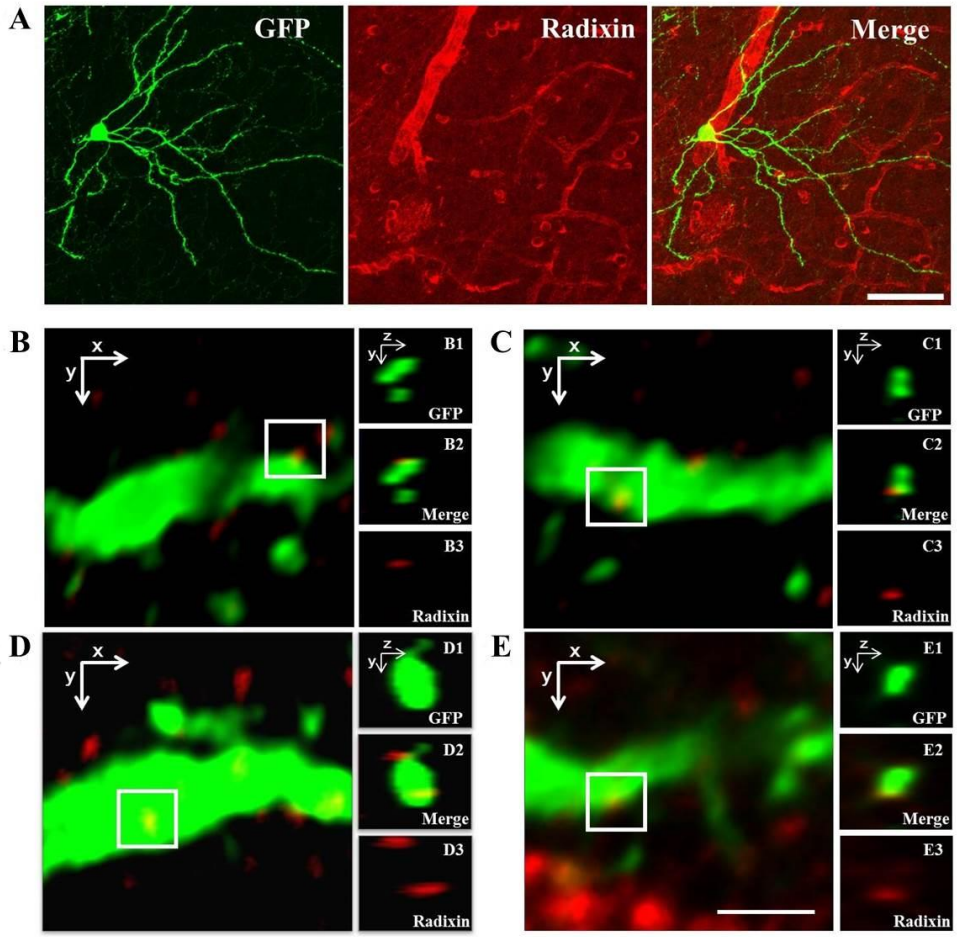


Figure 4. Visualization of the existence of radixin proteins on dendritic shafts of medium spiny neruons in the NAcc. (A) Representative low-magnification pictures show immunofluorescence of both eGFP and radixin expressed in MSN with antibodies against GFP and radixin respectively. (B through E) Double immuno-labeling of GFP, and radixin shows the localization of radixin positive clusters on eGFP-positive dendritic shafts. The photos in boxed areas in B through E were taken in orthogonal section mode (B1-B3, C1-C3, D1-D3, D1-D3 and E1-E3) to confirm the presence of immuno-positive clusters in close apposition to or on eGFP-positive dendritic shafts. Scale bars=50 μm (A), 1 μm (E).

2. Overexpression of radixin wild-type in the NAcc blocks the development of conditioned locomotor activity induced by AMPH during pre-exposure phase

During pre-exposure phase, all rats were measured for their locomotor response in the activity boxes for 1 hour following IP AMPH injection in Paired group, or IP saline injection in Unpaired and Control group within each conditioning block.

Among GFP rats, the 2-way repeated measures ANOVA with conditioning group as the between and block as the within factors on the 1-hour locomotor activity counts revealed a significant effect of conditioning group [$F_{2, 109}$=30.57, $p<0.001$] and group x block interactions [$F_{8, 109}$=11.70, $p<0.001$], while there was no significant effects of block [$F_{4, 109}$=1.22, $p=0.310$]. As expected, Paired group exposed to AMPH displayed greater locomotor response throughout the conditioning phase than Unpaired and Control group exposed to saline (Figure 5A).

Likewise, among Rdx-MUT rats, Paired group exposed to AMPH also displayed greater locomotor response throughout the conditioning phase than Unpaired and Control group exposed to saline ($p<0.01$, $p<0.001$, respectively, by *post hoc* SNK comparisons) (Figure 5B). Interestingly, however, these effects were not observed in Paired Rdx-WT rats (Figure 5C). Further, *post hoc* SNK comparisons within blocks between different types of virus injections in Paired groups revealed that the locomotor response in Rdx-WT rats was significantly lowered compared to GFP and Rdx-MUT rats (Figure 5D), indicating that the overexpression of Rdx-WT in the NAcc blocks the development of conditioned locomotion in this group.

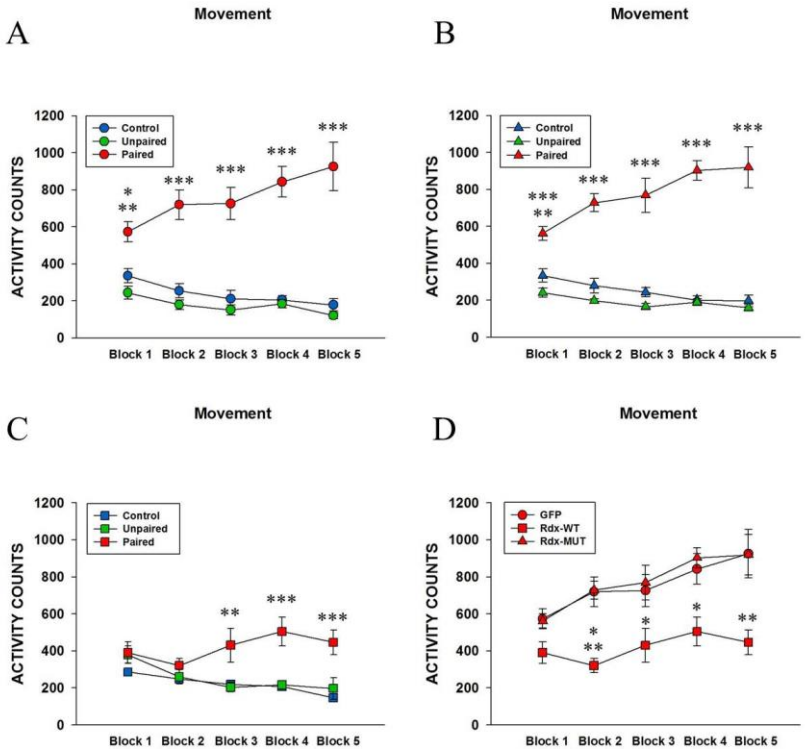


Figure 5. Overexpression of radixin wild-type in the NAcc blocks the development of conditioned locomotor activity induced by AMPH during pre-exposure phase. The locomotor activity during each conditioning block was counted following an IP AMPH injection in Paired group, and saline injection in Unpaired and Control group. Data are shown as group mean (+SEM) total locomotor activity counts during the 1-hour test. Numbers: Control-GFP (N=7), Unpaired-GFP (N=6), Paired-GFP (N=9), Control-Rdx-WT (N=6), Unpaired-Rdx-WT (N=6), Paired-Rdx-WT (N=5), Control-Rdx-MUT (N=5), Unpaired-Rdx-MUT (N=6), Paired-Rdx-MUT (N=7). Symbols indicate significant differences revealed by *post hoc* SNK comparisons following 2-way RM ANOVA. *p<0.05, **p<0.01, ***p<0.001 significantly more counts compared to Control and Unpaired group in (A, B, C) and *p<0.05, **p<0.01, significantly lower counts compared to GFP and Rdx-MUT group in (D).

3. Overexpression of radixin wild-type in the NAcc inhibits the expression of conditioned locomotion

To study the role of radixin in the expression of AMPH-induced conditioning, all rats were tested for their conditioned locomotor response in the activity boxes for 1 hour following IP saline injection after 1 week of the last conditioning block. The 2-way ANOVA with conditioning group as the between and virus type as the within factors on the 1-hour locomotor activity counts revealed a significant effect of conditioning groups [$F_{2, 56}$=34.03, $p<0.001$] and groups x virus interactions [$F_{4, 56}$=2.74, $p<0.05$], while there were no significant effects of virus type [$F_{2, 56}$=1.50, $p=0.234$]. As expected, among GFP rats, Paired showed increased conditioned locomotor activity compared to Unpaired or Control groups ($p<0.001$; *post hoc* SNK comparisons). Likewise, among Rdx-MUT rats, Paired also showed increased conditioned locomotor activity compared to Unpaired or Control groups ($p<0.001$; *post hoc* SNK comparisons). Interestingly, however, these effects were not present in Rdx-WT rats. *Post hoc* SNK comparisons between Paired groups revealed that the conditioned hyper-locomotion appeared in GFP and Rdx-MUT rats was significantly inhibited ($p<0.01$) in Rdx-WT rats. The remaining groups did not show any significant differences from each other (Figure 6).

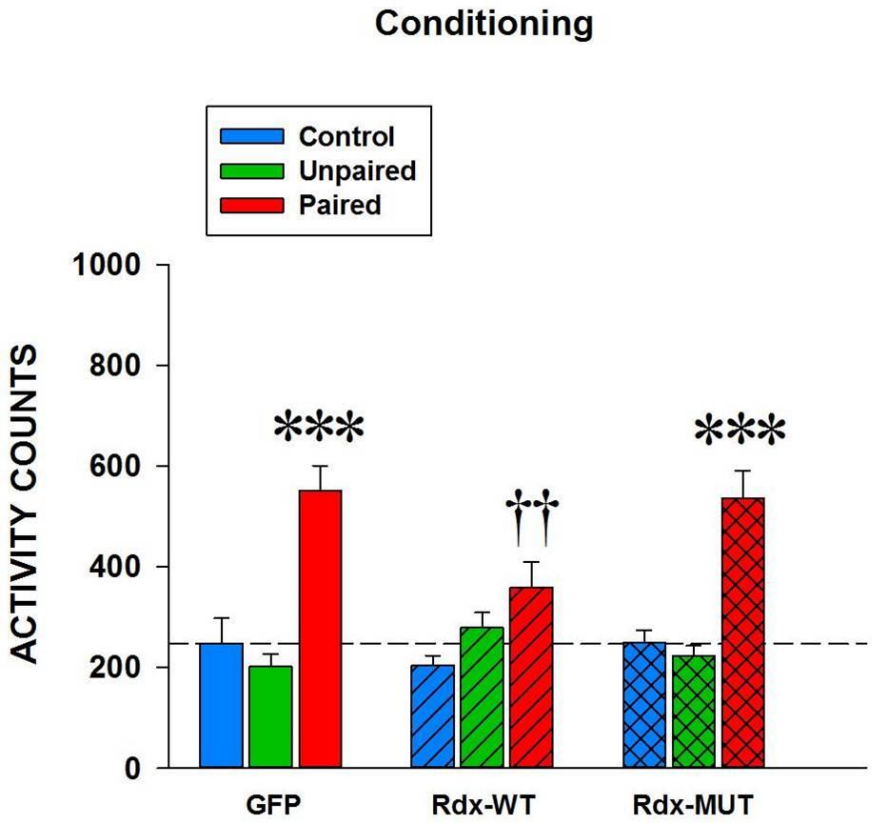


Figure 6. Overexpression of radixin wild-type in the NAcc inhibits the expression of conditioned locomotion. The conditioned locomotion was counted following an IP saline injection. Data are shown as group mean (+SEM) total locomotor activity counts during the 1-hour test. Numbers: Control-GFP (N=7), Unpaired-GFP (N=6), Paired-GFP (N=9), Control-Rdx-WT (N=6), Unpaired-Rdx-WT (N=6), Paired-Rdx-WT (N=5), Control-Rdx-MUT (N=5), Unpaired-Rdx-MUT (N=6), Paired-Rdx-MUT (N=7). Symbols indicate significant differences revealed by *post hoc* SNK comparisons following 2-way ANOVA. ***p<0.001, significantly more counts in Paired rats compared to those in the Control and Unpaired groups. †† p<0.01, significant difference between Paired rats when compared to GFP or Rdx-MUT.

4. Overexpression of radixin wild-type in the NAcc inhibits the expression of context-specific sensitized locomotion

To further study the role of radixin in the expression of context-specific sensitization induced by AMPH, AMPH challenge injection was followed 2 days after the conditioning test. The 2-way ANOVA with conditioning group as the between and virus type as the within factors on the 1-hour locomotor activity counts revealed a significant effect of conditioning groups [$F_{2, 56}$ =4.87, $p<0.05$] and virus type [$F_{2, 56}$ =3.56, $p<0.05$], while there were no significant effects of groups x virus interactions [$F_{4, 56}$ =2.48, $p=0.056$]. In GFP and MUT groups, Paired rats showed sensitized locomotor activity compared to Control group ($p<0.05$, $p<0.01$; *post hoc* SNK comparisons), while these effects were not present in Rdx-WT rats. *Post hoc* SNK comparisons between Paired groups revealed that there was a significant locomotor activity inhibition in Rdx-WT rats compared to GFP and Rdx-MUT rats ($p<0.01$) (Figure 7).

As expected, the locomotor response in Unpaired rats did not differ significantly from Control rats in all groups. This is result of so-called conditioned inhibition,[14] in which Unpaired group was under the strong influence of conditioned inhibitory properties of stimuli.[18] These results suggest that Rdx-WT overexpression in the NAcc affects the expression of sensitization in a context-specific manner.

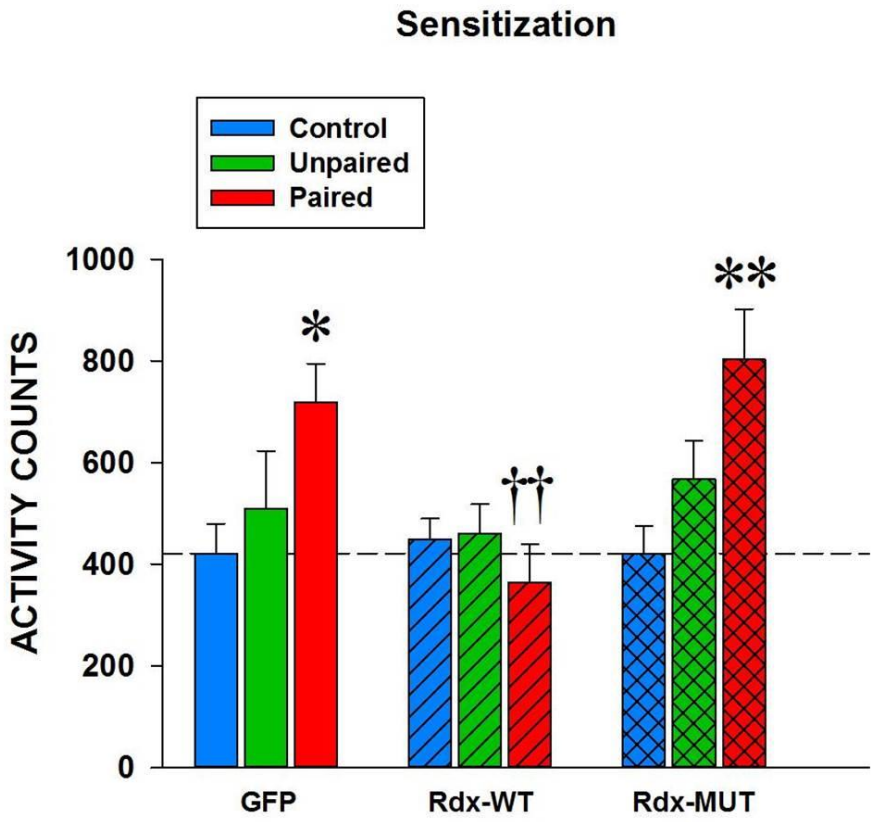


Figure 7. Overexpression of radixin wild-type in the NAcc inhibits the expression of context-specific sensitized locomotion. The sensitized locomotion was counted following an IP AMPH injection. Data are shown as group mean (+SEM) total locomotor activity counts during the 1-hour test. Numbers: Control-GFP (N=7), Unpaired-GFP (N=6), Paired-GFP (N=9), Control-Rdx-WT (N=6), Unpaired-Rdx-WT (N=6), Paired-Rdx-WT (N=5), Control-Rdx-MUT (N=5), Unpaired-Rdx-MUT (N=6), Paired-Rdx-MUT (N=7). Symbols indicate significant differences revealed by *post hoc* SNK comparisons following 2-way ANOVA. *p<0.05, **p<0.01, significantly more counts compared to Control group. ††p<0.01, significant difference between Paired rats when compared to GFP or Rdx-MUT.

5. Overexpression of radixin wild-type in the NAcc selectively inhibits the increase of dendritic spine counts depending on conditioning procedure

In order to examine whether there is any association between the behavioral effects and the number of dendritic spines regulated by radixin, all rats were perfused and the brains were collected 1 day after the sensitization test. Coronal sections of brain tissues including the NAcc were further processed for confocal imaging analysis and dendritic spines were counted (Figure 8 and 9).

A 2-way ANOVA conducted on total spine counts analysis revealed that there was a significant effect of conditioning groups [$F_{2,\ 48}$=6.49, $p<0.01$], while there were no significant effects in virus type [$F_{2,\ 48}$=1.54, p=0.23] and groups x virus interactions [$F_{4,\ 48}$=1.88, p=0.13]. *Post hoc* SNK comparisons in GFP group revealed that both Paired and Unpaired rats showed increased total spine density compared to Control ($p<0.05$). Similarly, *post hoc* SNK analysis in Rdx-MUT group indicated that total spine density was increased in Paired compared to Control rats, while there was marginally significant effect toward increase in Unpaired compared to Control group (p=0.059). Interestingly, in Rdx-WT group, while the total spine density was still maintained in Unpaired rats, these effects were no longer present in Paired rats. *Post hoc* SNK comparisons between Paired groups revealed that there was a significant decrease in total spine density in Rdx-WT rats compared to GFP ($p<0.05$) (Figure 10A).

When further examined for sub-types of spines, a similar pattern of the results was observed in thin spines as in total spine counts. A 2-way ANOVA conducted on thin spine counts analysis revealed that there was a significant effect of conditioning groups [$F_{2,\ 48}$ = 9.77, $p<0.001$], while there were no significant effects in virus type [$F_{2,\ 48}$ = 3.20, p=0.051] and groups x virus interactions [$F_{4,\ 48}$=2.60, p=0.05]. *Post hoc* SNK comparisons both in GFP and Rdx-MUT group revealed that Paired and

Unpaired rats showed increased thin spine density compared to Control ($p<0.05$, $p<0.01$). Interestingly again, in Rdx-WT group, while the thin spine density was significantly maintained in Unpaired rats, the thin spine density in Paired rats did not show significant difference compared to Control group. *Post hoc* SNK comparisons revealed that it was significantly decreased compared to GFP rats between Paired group ($p<0.05$) (Figure 10B). There were no significant differences found in other sub-types of spines (Figure 10C, D).

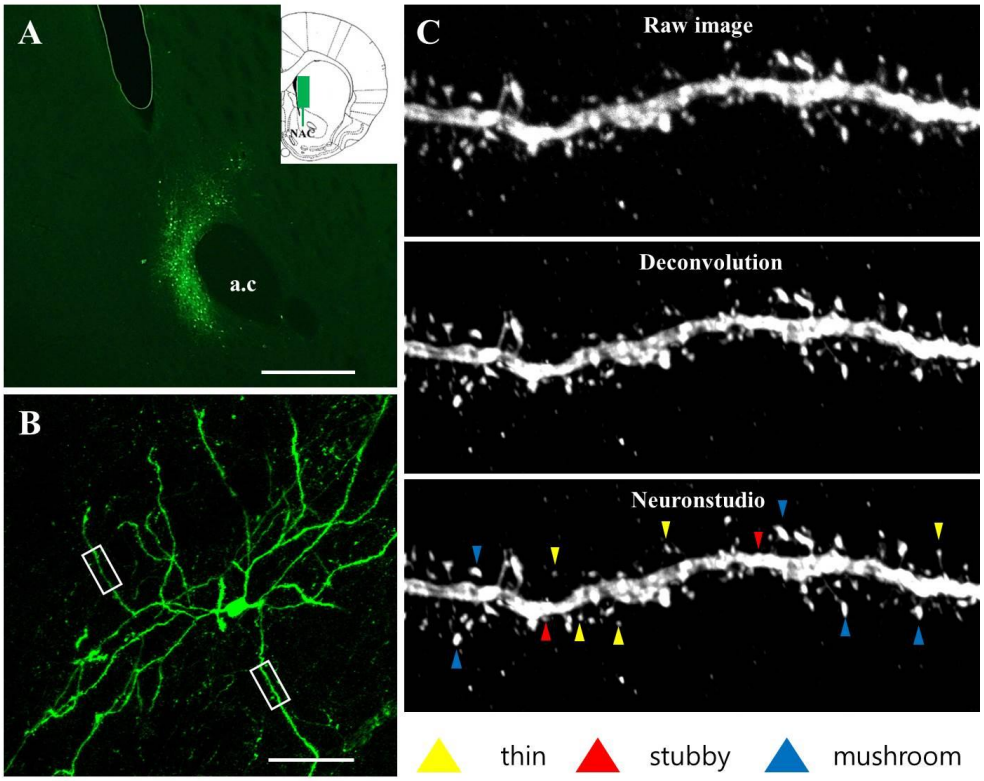


Figure 8. Images showing the copGFP-expressing MSNs in the NAcc and illustration of data processing. (A) A representative of epifluorescence image at low magnification shows target site of lentiviral vectors to the NAcc. (Upper right) Schematic diagram of the injection of AAV2-eGFP into the NAcc. (B) A confocal microscopy image shows a single MSN in the NAcc infected with lentiviral vectors and the white boxes indicates dendritic segments selected for spine analysis. (C) An illustration of data processing for spine analysis. Raw images were deconvolved with AutoQuant X3 deconvolution software to improve contrast and resolution. For quantitative spine analysis, neuronStudio software with rayburst algorithm was used to identify thin, stubby and mushroom dendritic spines in the NAcc. a.c, anterior commissure. Scale bars=500 μm (A), 50 μm (B).

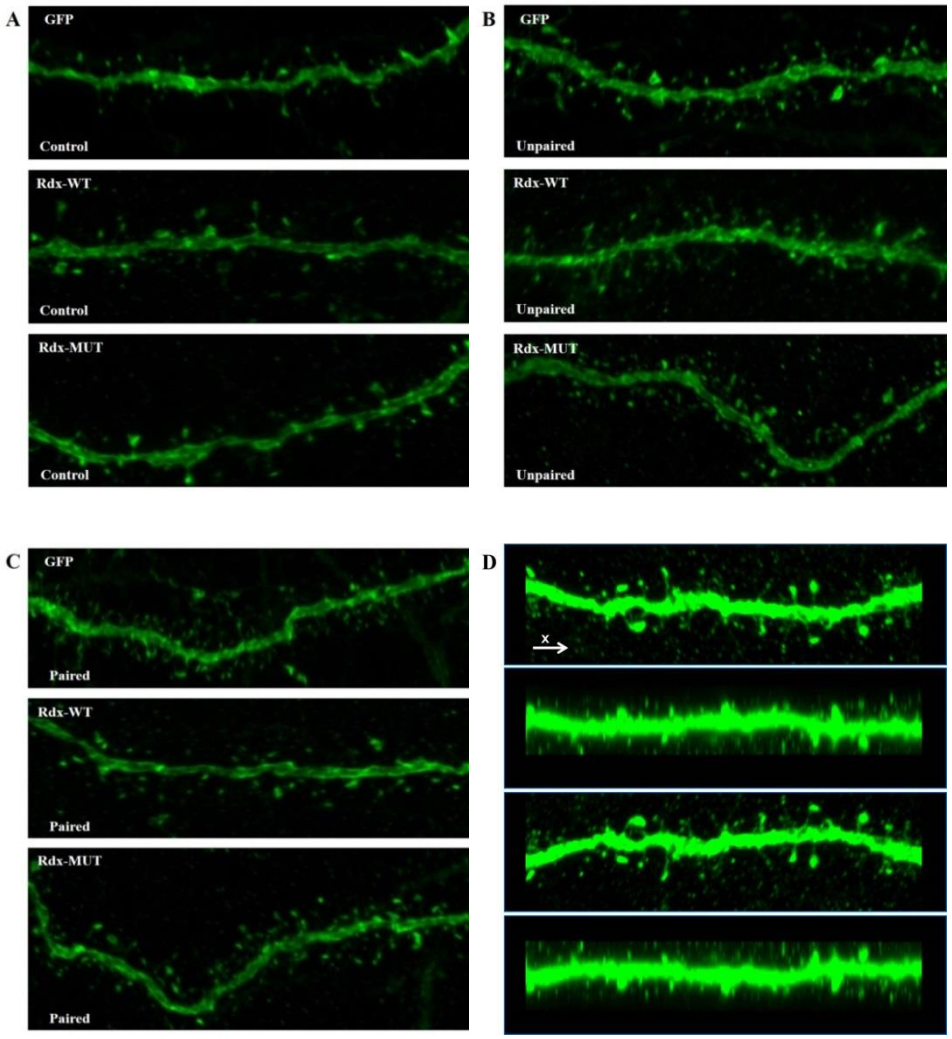


Figure 9. Representative images for dendritic spines of MSNs in the NAcc. (A) Representative high resolution images of dendrite segments in control group, (B) unpaired group and (C) paired group. Images are contrasted and shown in maximum intensity projections. The average length of dendritic segment traced for spine analysis is 35 μm (D) Serial images of dendritic segment were obtained by rotating the confocal image along X axis.

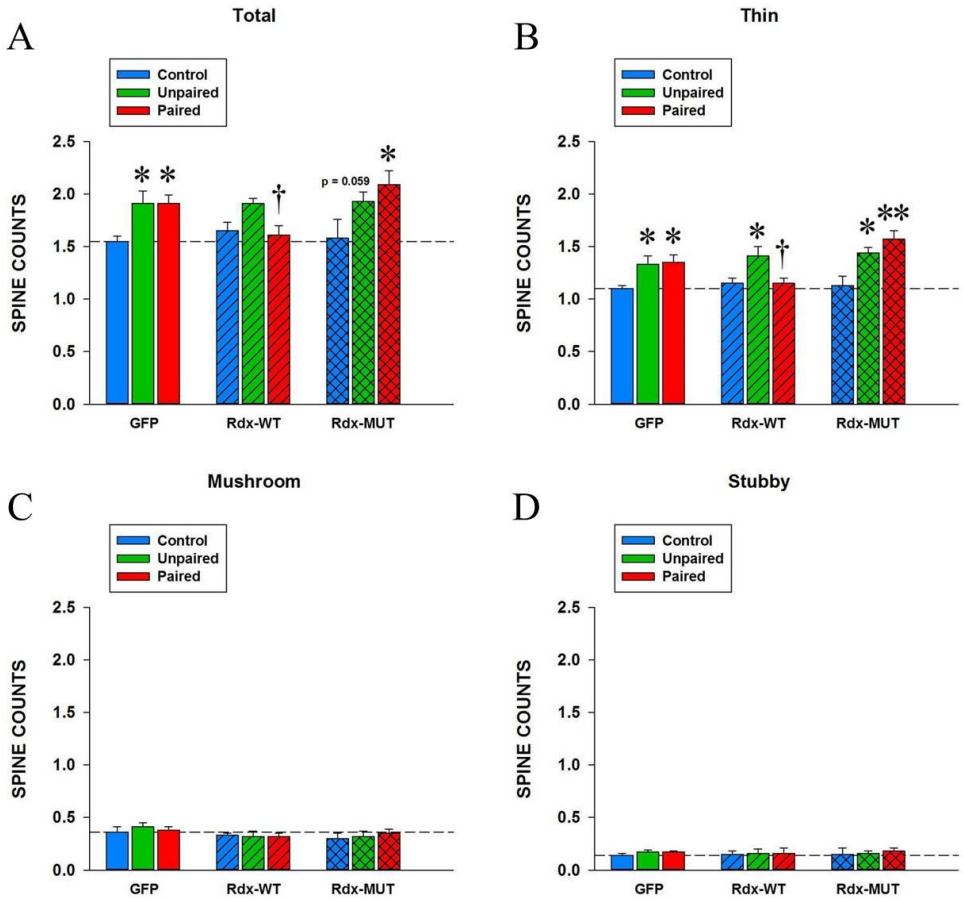


Figure 10. Overexpression of radixin wild-type in the NAcc selectively inhibits the increase of dendritic spine counts depending on conditioning procedure. Dendritic spine density was expressed as the number of spines normalized to 1 μm of dendritic length and data are shown as group mean (+SEM). Numbers: Control-GFP (N=6), Unpaired-GFP (N=6), Paired-GFP (N=8), Control-Rdx-WT (N=4), Unpaired-Rdx-WT (N=5), Paired-Rdx-WT (N=5), Control-Rdx-MUT (N=3), Unpaired-Rdx-MUT (N=5), Paired-Rdx-MUT (N=7). Symbols indicate significant differences revealed by *post hoc* SNK comparisons following 2-way ANOVA. (A) Total spine density, *p<0.05, significantly more counts compared to Control group. †p<0.05, significant difference between Paired rats when compared to GFP. In Rdx-MUT, there is a marginally significant effect toward increase in spine density in

Unpaired compared to Control rats (p=0.059). (B) Thin spine density, *p<0.05, **p<0.01, significantly more counts compared to Control group. †p<0.05, significant difference between Paired rats when compared to GFP. There were no significant differences found in both mushroom (C) and stubby (D) spine counts.

IV. DISCUSSION

It is thought that persistent maladaptive drug-related behaviors are associated with alterations in dendritic spines caused by drugs of abuse.[26] Previous findings show that the phosphorylation levels of ERM proteins are decreased by acute AMPH in the NAcc, suggesting that ERM proteins may play an important role in AMPH induced synaptic remodeling and subsequently following addictive behaviors.[35]

Before investigating the role of radixin in the NAcc in response to drugs of abuse, it was first examined the subcellular localization of radixin on MSNs of rat NAcc. Previously, it has been shown that ERM proteins are expressed in the brain, most abundantly in neurogenic regions.[45] The data presented here showed that radixin is expressed not only in SVZ but also in the NAcc. The presence of radixin and its subcellular distribution on MSNs of rat NAcc was directly showed in this thesis by combining AAV-eGFP expression system and high magnification confocal microscopy. As a result, it was observed that radixin clusters were in close apposition to membrane sides of dendrites, suggesting a specific role for radixin possibly contributing to new spine formation in response to drugs of abuse in the NAcc. Supporting this possibility, it has been previously shown that ERM proteins bind and interact with adhesion molecules such as telencephalin, which is known to be specifically present on the plasma membrane of neuronal dendrites and mediate dendritic filopodia formation.[46-49]

Next, it was examined whether radixin may contribute to the development of AMPH-induced associative memory (i.e., conditioning and context-specific sensitization) accompanied with structural plasticity of dendritic spines in the NAcc by manipulating its expression levels. As expected, the present findings have shown that Paired group of rats, with GFP alone, well developed conditioning when repeatedly paired with AMPH. This is also the case for Rdx-MUT, which indicates

that a pseudo active form of radixin does not disturb the development of new associative form of memory induced by AMPH pairing. Interestingly, however, over-expression of wild type radixin in the NAcc significantly disrupts the formation of new associative memory, as shown in Figure 6 that Paired group lost statistical significance compared to Control within Rdx-WT, while showed significant decrease of locomotor activity compared to both GFP alone and Rdx-MUT.

Repeated exposure to AMPH produces behavioral sensitization, a non-associative form of memory, with different mechanism separated and distinguished from the development of conditioning.[6,7] However, the expression of sensitization is also under the influence of environmental cues so that, depending on the conditioning procedure, it may be expressed as context-specific sensitization or conditioned inhibition.[6,7,14,15]

In fact, Figure 7 shows that, in both GFP and MUT groups, paired rats nicely expressed sensitized locomotor activity when AMPH was challenged, as a nice demonstration of an example of context-specific sensitization. Interestingly, however, these effects were absent in Paired group, with over-expression of wild type radixin in the NAcc. Considering the fact that cues within the environment where the drugs are repeatedly taken can gradually acquire rewarding effects of drugs by forming a new associative memory,[7] the absence of the expression of sensitized locomotor activity observed in Paired with Rdx-WT might be due to an inhibitory mechanism of over-expression of wild type radixin in the NAcc worked on a similar process that occurs during the development of conditioning. Again, these results suggest that over-expression of wild type radixin in the NAcc selectively disrupts the formation of new associative memory resulting in the blockade of the expression of conditioned locomotion and even further the expression of environment-specific sensitized locomotion.

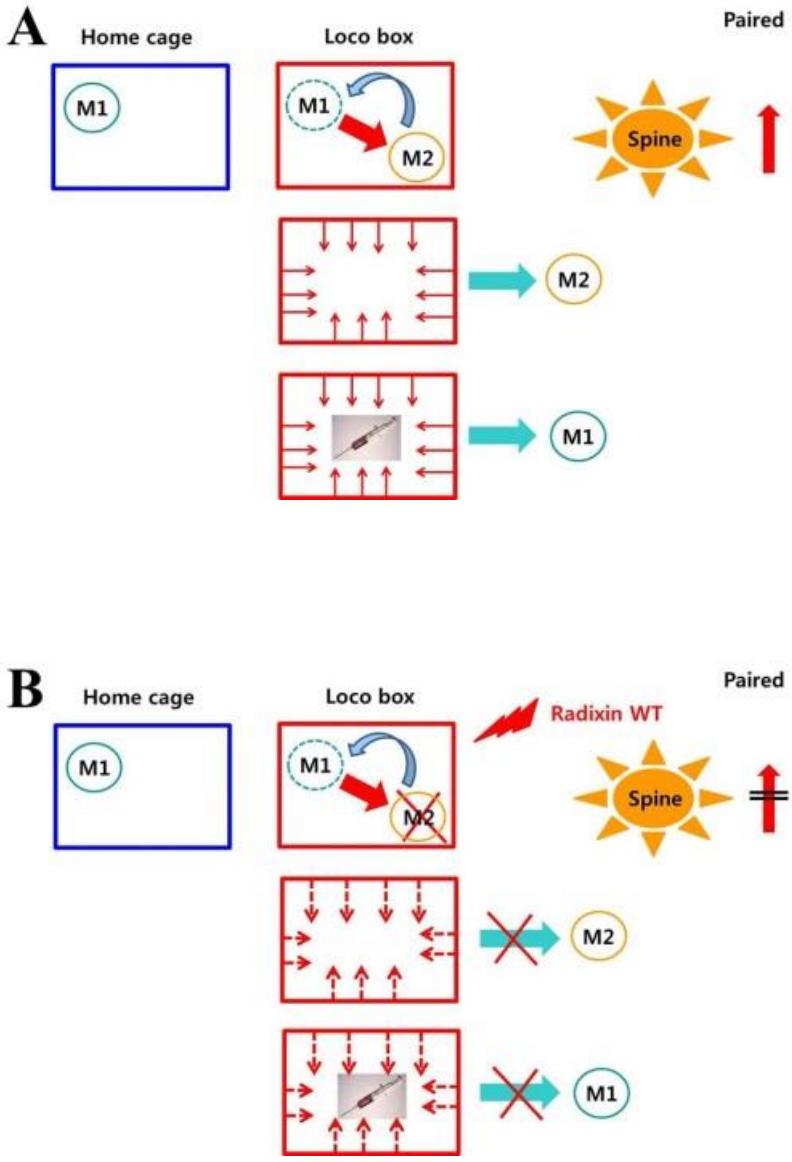
In addition, as expected, the expression of sensitization was not observed in Unpaired rats, even though they had previously received the same number and dose of AMPH like the Paired rats during the pre-exposure phase. This phenomenon has been called “conditioned inhibition”, in which the expression of sensitization was inhibited under the negative influence of drug-unpaired cues.[14,18] An important difference between Paired and Unpaired rats was the environments where they received AMPH. In Paired group, rats received AMPH in the test environment, while in Unpaired group, rats received AMPH in their homecage, each time during pre-exposure phase. After several pairings, cues within test environment can gradually acquire the ability of prediction for the presence or absence of drug in Paired and Unpaired groups, respectively. Thus, in the context of sensitization, it has been proposed that an inhibitory associative process can prevent the expression of sensitized locomotor response.[14] For example, it has been reported that cocaine seeking has been suppressed by conditioned inhibitors.[50] Interestingly, conceptually similar results have also been shown in humans; for example, cognitive factors such as belief about drug has a strong impact on subjective craving[51] and can modify neural activity related to cocaine in addicts.[52,53] In the present experiments, conditioned inhibition was observed in Unpaired groups with either GFP or Rdx-MUT, while it was not in Unpaired groups with Rdx-WT overexpression in the NAcc, indicating that both process (i.e., conditioning and context-specific sensitization) are distinctively controlled possibly by different mechanism.

As shown in the literature, it has been suggested that experience-dependent changes in behavior are associated with the structural plasticity of dendritic spines in relevant brain areas.[22-24] Similarly, drugs of abuse also induce structural plasticity of dendritic spines especially in the NAcc of brain reward circuitry, and it is thought to contribute to the development of drug addiction.[25-27] It was observed in the present study that repeated exposure to AMPH increased the number of total spine density in

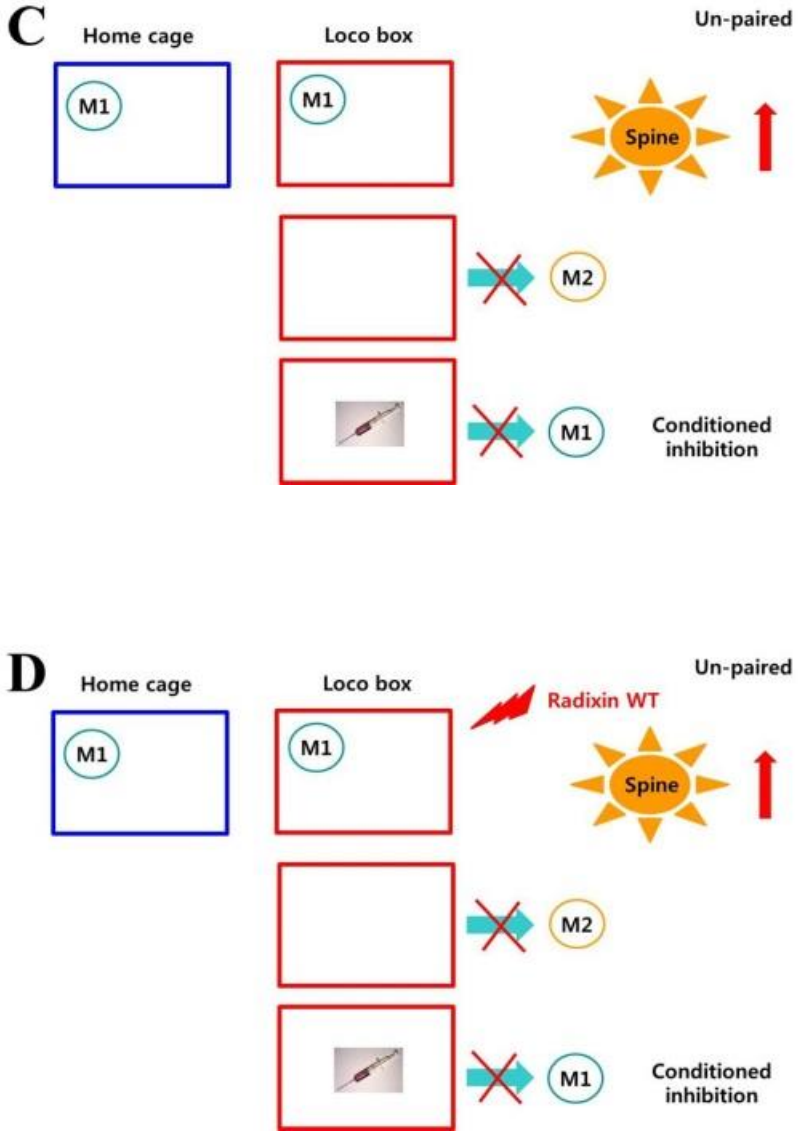
rats expressing GFP only in the NAcc both in Paired and Unpaired groups. These effects were also appeared in rats expressing Rdx-MUT in the NAcc both in Paired and Unpaired groups.

Interestingly, however, when wild-type radixin was over-expressed in the NAcc, only Paired group showed significantly lowered spine counts compared to GFP only ($P=0.034$) and Rdx-MUT ($P=0.004$), while Unpaired remained higher. Further analysis for the sub-types of spines revealed that thin spines mostly contributed to the increase of total spine density, while stubby and mushroom spines had no effects. The very similar pattern of results for the spine density was observed in thin spines as in total, indicating that thin spine is the most important major contributor to the change of total spines. The morphology of spines often reflects their stability. For example, it is known that mushroom spines are functionally and structurally stable, containing more α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, and conventionally thought as memory spines.[54] Whereas thin spines mostly contain *N*-methyl-D-aspartate (NMDA) receptors with only a few AMPA receptors in their heads and preferentially respond to afferent stimulus.[55] Thus, the increase in thin spine density may be able to contribute to the increase of the potential plastic sites upon subsequent stimulus.[56] In the present findings, the increase of thin spine density in the NAcc may reflect the increase of neuronal substrates required to store newly formed memory evoked by drug-related stimuli. Interestingly, these morphological observations for dendritic spines were correlated well with behavioral outcomes, suggesting that structural plasticity for dendritic spines may mediate the appearance of addictive behaviors. If so, the findings that the over-expression of wild-type radixin in the NAcc inhibits the increase of thin spine density only in Paired, while saves in Unpaired, may indicate that radixin in this site importantly contributes to the formation of thin spines, especially related with context-specific associative memory (Figure 11).

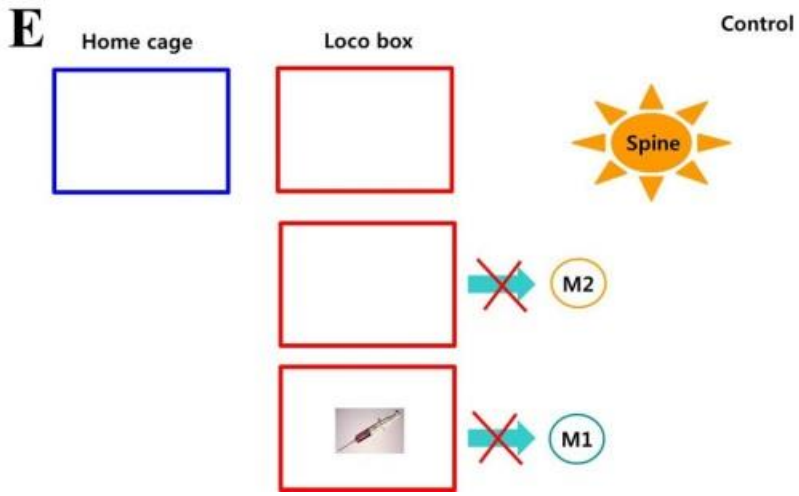
Signaling pathways involved in dendritic spine regulation are so complicated and little information is available on what aspects of ERM proteins are involved in dendritic spine plasticity in the brain. However, in vitro evidence has provided some clues to the possibility that activated ERM proteins are involved in dendritic spine formation. For example, it has been shown that nerve growth factor (NGF) induced PC12 cell neurites outgrowth required the phosphorylation of moesin at Thr558.[57] Also in cortical neurons, the increase of phosphorylation levels of moesin at Thr 558 has been shown to be associated with increased dendritic spine density, while interrupting the endogenous moesin by using shRNA silencing method abrogates this spinogenesis in vitro.[58] Similarly, in the present study, activity dependent up-regulation of activated radixin protein levels would be important and may direct the signaling pathways to contribute to dendritic spine formation. In that sense, a pseudo-active mutant radixin, Rdx-MUT, lies in the same direction with endogenously activated radixin, by which rats with Rdx-MUT may manifest same results as shown in those with GFP alone for both behavior and spine counts in the present findings. In contrast, over-expression of radixin wild-type may interrupt the balance between active and inactive form of ERM proteins, thereby bringing the weight toward “inactivation”, resulting in the blockade of new spine formation during the context-specific associative memory formation between drug and environment.

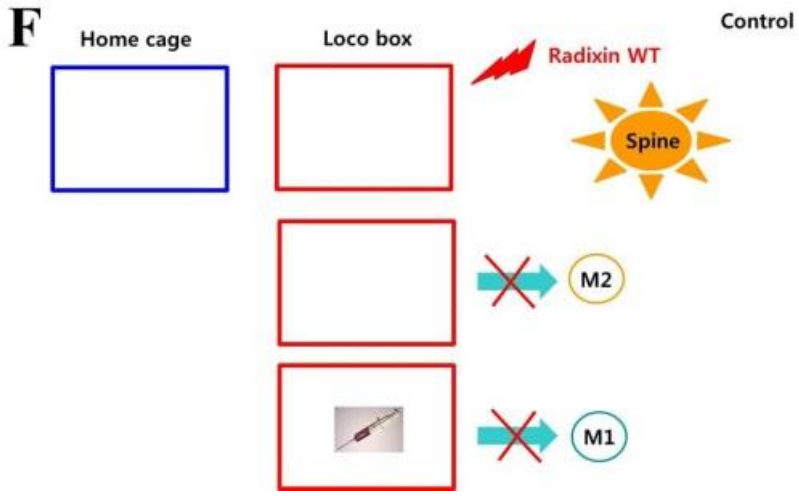


M1 (non-associated memory by drug only)
M2 (associated memory by drug and environment pairing)



M1 (non-associated memory by drug only)
M2 (associated memory by drug and environment pairing)





M1 (non-associated memory by drug only)
M2 (associated memory by drug and environment pairing)

Figure 11. A hypothetical diagram showing that the radixin in the NAcc specifically contributes to the development of AMPH-induced associative memory. Repeated exposure to AMPH in the locomotor acitivity box (test environment) induced associative changes in neural systems that mediate M2 in paired rats, thus context can elicit a drug-like conditioned response when the rats re-exposed to the environment. Repeated exposure to AMPH also sensitized a neural substrate mediating M1 which reflected as an enhanced psychomotor activation. There is also an increase in total spine density in the NAcc of paired rats (A). With the overexpression of Rdx-WT in the NAcc, the formation of new spines was inhibited, and consequently impaired the M2 resulting in the blockade of expression of conditioned locomotion, and even further impaired M1, i.e. the expression of context-specific sensitized locomotion (B). In Unpaired rats, repeated exposure to AMPH in the homecage induced M1 and new spine formation in the NAcc but not M2 in test environment. Although, this group of rats may have developed M1, the expression of sensitization was not appeared in test environment where they have never received the AMPH. This phenomenon is called conditioned inhibition by which inhibitory associative stimuli that are specifically predict the absence of drug can prevent the expression of sensitized locomotor response. (C). In this group, Rdx-WT overexpression in the NAcc did not affect both new spine formation and the conditioned inhibition, which may be controlled distinctively from context-specific sensitization appeared in Paired (D). Acute AMPH exposure could not induce drug related memories and new spines in the NAcc of control rats (E). Rdx-WT overexpression had no effects on behavioral and morphological observations in control rats (F).

V. CONCLUSION

1. Over-expression of wild type radixin in the NAcc selectively disrupts the formation of new associative memory resulting in the blockade of the expression of conditioned locomotion and even further the expression of context-specific sensitized locomotion.

2. The increase of total (esp. thin) spine density in the NAcc may reflect the increase of neuronal substrates required to store newly formed memory evoked by drug-related stimuli.

3. Over-expression of wild-type radixin in the NAcc inhibits the increase of thin spine density only in Paired, while saves in Unpaired, which may indicate that radixin in this site importantly contributes to the formation of thin spines, especially related with context-specific associative memory.

4. The present study is the first direct demonstration, to my knowledge, that radixin in the NAcc specifically contributes to the development of AMPH-induced context-specific associative memory (i.e., conditioning and context-specific sensitization), and it does so by regulating structural plasticity of dendritic spines in this site.

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ABSTRACT (in Korean)

암페타민으로 유도한 보행성 조건화 반응에서 중격측좌핵 내 래딕신의 역할

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채 문 정

중격측좌핵은 보상회로라고 불리는 신경회로망에서 중독성 약물의 효과를 매개하는 중요한 영역이다. 실험동물에게 정신자극제를 반복하여 주면 중격측좌핵 내 신경세포 수상돌기 가시의 구조적 변화를 초래하는데 이러한 변화들은 정신자극제로 인한 행동과민반응과 조건화 반응과 연관되어 있다고 보도 된 바 있다. 수상돌기 가시는 중요한 시냅스후 구조로 축삭종말로부터 오는 전기적신호를 매개하는데 구조적 모양에 따라 thin, stubby, mushroom 종류로 분류되어지기도 한다. 수상돌기 가시의 구조적 가소성은 그 속에 존재하는 액틴미세섬유의 재구성에 의거하는데 이 또한 수많은 액틴미세섬유를 조절하는 단백질들의 조절 하에 이루어진다. 높은 상동성을 보이는 Ezrin-Radxin-Moesin (ERM) 단백질은 세포 내에서 구조단백질인 섬유성 액틴 단백질을 원형질 막과 연결 시켜주는 역할을 한다고 알려져 있다. 뇌에서도 관찰된 바가 있는 ERM단백질은 최근 수상돌기 가시의 형성에 연관되어 있다는 결과들이 발표되고 있다. 이러한 지식적 배경은 ERM단백질이 정신자극제로 인한 수상돌기 가시의 구조적 변화와 연관성이 있음을 시사한다. ERM단백질이 뇌에서 발현된다는 연구결과는 있으나 중격측좌핵 신경세포 내 분포에 대해서는 알려진 바가 많이 없다.

이에 따라 본 연구에서는 먼저 녹색형광단백의 세포 내 발현, 면역형광기법, 공초점 레이저 주사 현미경 등 방법을 통하여 ERM단백질 중의 하나인 래딕신의 중격측좌핵 신경세포 내 분포를 밝혔고 더 나아가 중격측좌핵 내 래딕신이 암페타민으로 유도한 행동과민반응과 보행성 조건화 반응에 미치는 영향을 알아보았고 이때 래딕신이 중격측좌핵 내 수상돌기 가시의 변화에 미치는 영향도 관찰하였다.

우선, Green Fluorescent Protein (GFP), Radixin wild-type (Rdx-WT), pseudo-active radixin mutant T564D (Rdx-MUT) 유전정보를 함유한 렌티바이러스를 입체 정위시술을 통하여 쥐들의 중격측좌핵 내에 국소 주입하였다. 2주간의 회복기간을 거친 쥐들은 다시 세 그룹으로 나누어졌고 다음과 같은 과정으로 약물과 환경을 짝지어 주었다. Paired 그룹은 첫째 날에 암페타민(1 mg/kg, IP)을 보행행동 측정상자에서 주고 둘째 날에 사육상자에서 생리식염수를 주었으며, Unpaired 그룹은 첫째 날에 보행행동 측정상자에서 생리식염수를, 둘째 날에 사육상자에서 암페타민(1 mg/kg, IP)을 주었으며, 마지막으로 Control 그룹은 첫째 날과 둘째 날에 두 환경에서 모두 생리식염수를 준 그룹으로, 앞의 과정을 총 5회 반복하여 실시하였다. 이들 쥐는 1주일간의 약물중지기간을 거친 후, 모든 쥐에 생리식염수를 복강 주입하고 보행행동 측정상자에서 1시간 동안 조건화에 의한 보행성 행동반응을 측정하였고, 이틀 뒤 모든 쥐에 암페타민(1 mg/kg)을 복강 주입하고 보행행동 측정상자에서 1시간 동안 행동과민반응을 측정하였으며, 중격측좌핵 내 수상돌기 가시의 변화를 관찰하기 위하여 다음 날 모든 쥐를 관류 고정하였다. 그 결과, Paired 그룹에서 중격측좌핵 내에 GFP와 Rdx-MUT 단백질을 과발현시킨 쥐들은 조건화 행동반응과 행동민감성반응을 보였으나, Rdx-WT 단백질을 중격측좌핵 내에 과발현한 Paired 그룹 쥐들에게는 이러한 증가 효과가 억제되는 것을 관찰하였다. 뿐만 아니라, 중격측좌핵 내 Rdx-WT 단백질의 과발현은 약물과 짝지어진 Paired 그룹 쥐들의 수상돌기 가시의 형성도 억제하였다. 하지만 이러한

중격측좌핵 내 Rdx-WT 단백질의 효과는 Unpaired 그룹 쥐들의 암페타민으로 인한 수상돌기 가시의 형성에는 영향이 없는 것으로 나타났다. 이 결과를 종합하면, 중격측좌핵 내 래딕신은 환경특이적 연합학습과 연관된 수상돌기 가시의 형성을 통하여 약물과 짝지어진 환경에 대한 조건화 반응 및 환경특이적 행동과민반응의 형성에 기여하는 것으로 사료된다.

핵심되는 말: 래딕신, 중격측좌핵, 보행성 조건화 반응, 수상돌기 가시, 암페타민

PUBLICATION LISTS

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