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Repeated exposure to methiopropamine increases dendritic spine density in the rat nucleus accumbens core

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ABSTRACT

Repeated exposure to classical psychomotor stimulants, like amphetamine (AMPH), produces locomotor sensitization and accompanied structural plasticity of dendritic spines in the nucleus accumbens (NAcc). Following our previous report that repeated administration of methiopropamine (MPA), a structural analog to meth-AMPH, produces locomotor sensitization, it was examined in the present study whether this behavioral change also accompanies with structural plasticity in the NAcc in a similar way to AMPH. A week after adeno-associated viral vectors containing enhanced green fluorescent protein (eGFP) were microinjected into the NAcc core, rats were repeatedly injected with saline, AMPH (1 mg/kg, IP), or MPA (5 mg/kg, IP) once every 2–3 days for a total of 4 times. Two weeks after last injection, all rats were perfused and their brains were processed for immunohistochemical staining. The image stacks for dendrite segments of medium spiny neuronal cells in the NAcc core were obtained and dendritic spines were quantitatively analyzed. Interestingly, it was found that the number of total spine density, with thin spine as a major contributor, was significantly increased in MPA compared to saline pre-exposed group, in a similar way to AMPH. These results indicate that MPA, a novel psychoactive substance, has similar characteristics with AMPH in that they both produce structural as well as behavioral changes, further supporting MPA's dependence and abuse potential.

1. Introduction

Methiopropamine (MPA, 1-(thiophen-2-yl)-2-methylaminopropane), a novel psychoactive substance, is a structural analog to a classical psychostimulant, methamphetamine, in which the benzene ring was replaced by a thiophene ring (Iversen et al., 2013; Lee et al., 2014; Welter-Luedeke and Maurer, 2016). Although its use as a recreational drug has been increasing around the world in recent years (United Nations Office on Drugs and Crime, 2013, 2015), there are no proper regulations yet for its transactions in most countries. However, as its recreational use has been reported to produce acute toxicity and even death (Lee et al., 2014; Anne et al., 2015), the increasing concerns for MPA abuse have been raised and consequently lead the World Health Organization to produce a Critical Review Report in the year of 2016, in which it was designated as a novel drug with dependence and abuse potential (Methiopropamine Critical Review Report, 2016).

It is well-known that psychomotor stimulants such as amphetamine (AMPH) produce locomotor sensitization, when repeatedly administered to rodents (Vezina, 2004). As sensitization, once developed, remains as a long-term memory contributing to the animal's addictive behaviors (Anagnostaras et al., 2002), it is widely used as a form of animal model for escalating drug use and long-lasting craving observed in human addicts (Robinson and Berridge, 1993; Vezina, 2004). Similar to AMPH, it has been previously shown in our own laboratory that repeated administration of MPA in rats also produces locomotor sensitization (Yoon et al., 2016), supporting its dependence and abuse potential (Methiopropamine Critical Review Report, 2016).

Considering that locomotor sensitization remains for a long period of time as a form of long-term memory (Anagnostaras et al., 2002), it might be natural to expect to observe that there may be long-lasting neuronal adaptations accompanying structural plasticity within the brain reward circuitry, especially in the nucleus accumbens (NAcc), which is an important neuronal substrate mediating the rewarding and locomotor activating effects of drugs of abuse (Robbins et al., 1989; Goto and Grace, 2008). Interestingly, there have been some reports in the rat that psychomotor stimulant-induced locomotor sensitization is associated with the increase of dendritic spine densities for the medium spiny neurons (MSNs) in the NAcc (Robinson and Kolb, 1999; Li et al., 2003), especially in the core (Li et al., 2004; Nordquist et al., 2008; Christian et al., 2017) among the two sub-regions of the NAcc.

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Although it remains to uncover the causative linkage between structural plasticity and addictive behavior (Russo et al., 2010), it is reasonable to assume that the change of dendritic spine density reflects some critical changes of neurophysiological and chemical properties in this site, mediating addictive behaviors (Brown et al., 2011; Wang et al., 2013; Chidambaram et al., 2019).

Although MPA has shown to have an abuse potential to induce locomotor sensitization in the rat similar to AMPH (Yoon et al., 2016), it has not been determined yet whether it might also induce structural plasticity in the NAcc. Thus, in the present study, we examined the dendritic spines in the NAcc, especially in the core, following MPA-induced sensitization development.

2. Materials and methods

2.1. Subjects and drugs

Male Sprague-Dawley rats weighing 220–250 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 h light/dark cycle room (lights out at 8:00 pm), and all experiments were conducted during the day time. The rats had access to food and water *ad libitum* at all times. All animal use procedures were conducted according to an approved Institutional Animal Care and Use Committee protocol.

MPA hydrochloride was synthesized and kindly provided by Professor Young Sup Lee at the Department of Pharmacy, Kyung Hee University (Seoul, South Korea). It was dissolved to a final working concentration of 5.0 mg/ml in sterile 0.9% saline. Dextroamphetamine sulfate (U.S. Pharmacopeia, Rockville, MD) was dissolved to a final working concentration of 1.0 mg/ml in sterile 0.9% saline. The doses of MPA and AMPH were chosen based upon our own previous reports (Yoon et al., 2016; Jang et al., 2018).

2.2. Surgery for virus injection

Rats were anesthetized with intraperitoneal (IP) ketamine (100 mg/kg) and xylazine (6 mg/kg) and placed in a stereotaxic instrument with the incisor bar at 5.0 mm above the interaural line. Infusion cannulas (28 gauge; Plastics One, Roanoke, VA) connected to 2 μℓ Hamilton syringes (Reno, NV) via PE-20 tubing were angled at 10° to the vertical and bilaterally 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 virus (serotype 2) particles containing enhanced green fluorescent protein (eGFP-AAV2) (Vector Biolabs, PA) were then bilaterally infused for 1 min and another 10 min allowed for diffusion before the infusion cannulas were raised. After viral infection was finished, the incised skin covering the skull was grabbed with surgical staplers. Then, rats were returned to their home cages for one week of recovery period.

2.3. Locomotor activity

Locomotor activity was measured with a bank of 9 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. Two infrared light photobeams (Med Associates, St. Albans, VT), positioned 4.5 cm above the floor and spaced evenly along the longitudinal axis of the box, were used to estimate horizontal locomotor activity.

2.4. Tissue preparation and immunohistochemistry

After 2 weeks of drug-free withdrawal period, rats were all deeply anesthetized with ketamine (100 mg/kg) and xylazine (6 mg/kg) and then perfused transcardially with 10 mM phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde solution in 10 mM PBS (pH 7.4). Brains were removed and coronal cut was made into blocks. The tissue blocks containing the NAcc region were transferred to ice-cold 4% paraformaldehyde in 10 mM PBS (pH 7.4) for another 6 h. Then, blocks were washed with 10 mM PBS, cryoprotected in 30% sucrose solution and stored at −80 °C.

Free-floating 100 μm sections from frozen tissue blocks were prepared on a cryostat. They were immersed for 1 h in 10 mM PBS containing 5% normal goat serum (Jackson Immunoresearch, PA) and 0.3% Triton X-100. Then, they were incubated overnight with anti-GFP antibodies (chicken polyclonal, 1:1000, Abcam) diluted in 10 mM PBS containing 2% normal goat serum and 0.1% Triton X-100 at 4 °C. Following overnight incubation, the sections were rinsed 3 times in 10 mM PBS containing 0.1% Triton X-100 and incubated with the anti-chicken secondary antibody coupled to Alexa 488 (1:1000; Invitrogen) for 2 h at room temperature. They were rinsed again and cover-slipped with Vetashield mounting medium (Vector Laboratories, UK).

2.5. Imaging analysis for spine counts

To acquire enhanced GFP signals from AAV infected neurons, GFP staining was done in 100 μm tissue sections. This approach has been applied to increase the brightness of sub-micron dendritic spines in infected neurons (Wang et al., 2013; Fakira et al., 2016). For spine analysis, we imaged individual dendritic segments of medium spiny neurons (MSNs) following suggested requirements as in a previous publication (Cahill et al., 2018) [i.e., (i) the segments have to show uniform GFP distribution, (ii) the segments cannot be overlapped with other neighboring dendritic segments and be traced back to their soma, (iii) secondary and tertiary dendritic segments at least 50 μm from soma should be chosen].

All images were acquired under a LSM700 confocal laser scanning microscope (Carl Zeiss) with a 488 nm argon laser. For whole cell reconstructions, confocal stacks of MSNs were imaged at 20× air objective lens (numerical aperture 0.7) with a z-step size of 1.3 μm and an XY resolution of 0.625 μm. For dendrite imaging, dendritic segments stacks spaced 0.1 μm were acquired with a 63× oil-immersion objective (numerical aperture 1.4) and a scan zoom of 2.5. The pinhole aperture set to 1 Airy Unit and the line average of 4 was used. The full dynamic ranges of images were obtained by adjusting the laser intensity and photomultiplier tube 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 0.04 × 0.04 × 0.1 μm^3 in x-y-z plane.

To improve contrast and resolution, raw confocal images were deconvolved with AutoQuant X3 deconvolution software. Dendrite tracing and automatic spine detection was then performed using NeuronStudio software (courtesy of Icahn School of Medicine at Mount Sinai, New York) with rayburst algorithm (Rodriguez et al., 2008), which classifies spines into thin, mushroom or stubby according to the classification dimensions (e.g., head to neck ratio, head diameter). The minimum and maximum values for spine height were set at 0.5 μm and 3.0 μm, respectively. For minimum stubby size, 22 voxels were set based on published criteria (Jung et al., 2013).

2.6. Design and procedures

Upon arrival, all rats passed a week-long adaptation period to the new housing environment. Then, virus injection surgery was followed. Once they were recovered from surgery, rats were randomly assigned to three groups and pre-exposed to saline, MPA (5.0 mg/kg, IP), or AMPH (1.0 mg/kg, IP) with a total of four injections 2–3 days apart. This regimen of drug injection with doses for drugs that we used was previously shown to produce locomotor sensitization (Yoon et al., 2016;

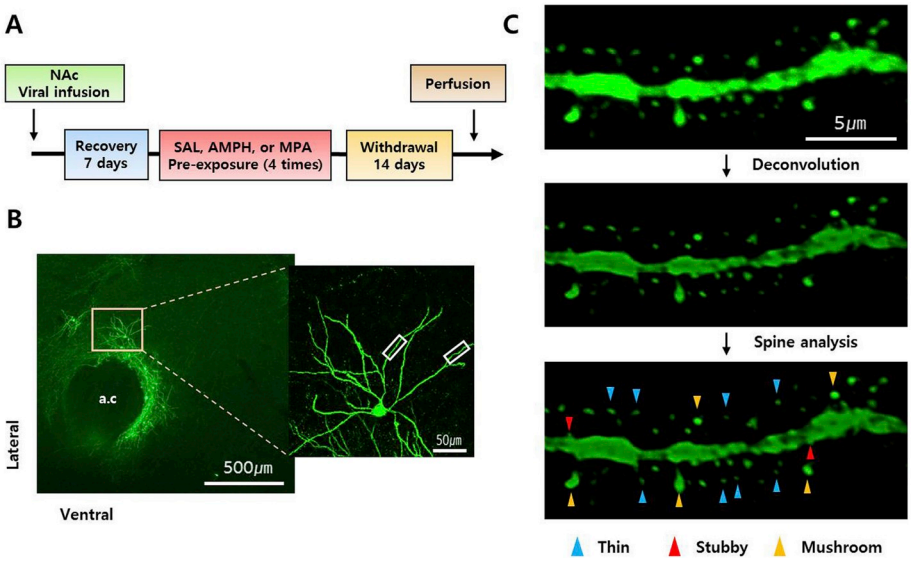

Fig. 1. Illustrations of eGFP-expressing neurons and dendritic segments following AAV infection in the NAcc core. (A) Time lines for the experimental procedures. (B) A representative low magnification (4×) epifluorescence image of MSNs expressing AAV-mediated eGFP and a higher magnification (20x) confocal microscopy image of a single MSN in the NAcc core. The white boxes represent dendritic segments selected for analysis. (C) An example of a dendritic segment image deconvolved and used for spine analysis using NeuronStudio.

Jang et al., 2018). To avoid any confounding effects of conditioning, rats were administered with drugs in different places (i.e., in the activity boxes for the first and the fourth injections and in their home cages for the other injections) (Kim et al., 2001; Song et al., 2013). Locomotor activity was measured only in days 1 and 4, in which they were first habituated to the activity boxes for 1 h, and their locomotor activity was measured for 1 h immediately following saline, MPA, or AMPH IP injections. Two weeks after the last pre-exposure injection, all rats were perfused and their brains were removed. Using immunohistochemistry and confocal imaging, spine analyses were conducted by experimenter who knew only tissue numbers, but was blind to what group they belong. A total of 15 rats were used and included in the statistical analysis in this study. Fig. 1A shows an outline of the whole experimental procedures.

2.7. Statistical analyses

Statistical analyses were performed using the Sigma Plot version 12.0 (Systat Software, San Jose, CA). The locomotor activity counts were analyzed with two-way repeated analysis of variance (ANOVA), while the spine density was analyzed with one-way ANOVA, followed by post-hoc Tukey comparisons. Differences between experimental conditions were considered statistically significant when $p < 0.05$.

3. Results

3.1. MPA produces increase of locomotor activity similar to AMPH

As expected, MPA produced increased locomotor activity similar to AMPH during drug pre-exposure (Table 1). The two-way repeated measures ANOVA conducted on the 1 h total locomotor activity counts, on both day 1 and day 4, revealed a significant effect of drugs [$F_{2,12} = 10.87$, $p < 0.01$], while there was no significant difference detected between days. The drug treatment regimen we used in the present study has been shown in our previous findings (Yoon et al., 2016) to produce locomotor sensitization for MPA when challenged with the same dose (5.0 mg/kg) after 2 weeks of withdrawal period. However, in the present study, it was not measured further in order to examine spine status before challenge injection.

3.2. Repeated injection of MPA produces increase of spine density in the NAcc core similar to AMPH

After 2 weeks of drug-free withdrawal period, different sub-types of dendritic spine morphology and their densities were analyzed for neuronal cells in the NAcc core. Fig. 1B shows a representative image of eGFP-AAV infected MSNs in the NAcc core. For spine analysis, dendritic segments were imaged via high resolution confocal microscopy. Raw 3D confocal images were then deconvolved and the density of total, thin, mushroom, and stubby spines were semi-automatically quantified using NeuronStudio software (Fig. 1C). We examined a total of 24–26 neurons per group (5 rats per group, 4 to 6 neurons per rat, 1 to 3 dendrites per neuron).

The one-way ANOVA conducted on these data showed significant effects between groups [$F_{2,\ 12} = 7.46$, $p < 0.009$]. Post hoc Tukey comparisons revealed that both MPA ($p < 0.05$) and AMPH ($p < 0.01$) compared to saline pre-exposure produced significantly increased total spine densities of MSNs in the NAcc core (Fig. 2). Further, we found that the increases of total spines were mainly contributed by the selective induction of thin spines in both MPA and AMPH pre-exposed groups [$F_{2,\ 12} = 7.56$, $p < 0.009$]. There were no significant changes observed in mushroom and stubby spine densities.

Table 1
Locomotor activity counts during pre-exposure period.

Pre-exposure	DAY 1	DAY 4
SAL (5)	35 ± 15	25 ± 5
MPA (5)	280 ± 77*	372 ± 95**
AMPH (5)	274 ± 88*	413 ± 56**

All rats were habituated for 1 h and their locomotor activity measured for an additional 1 h following their respective injections. Only at day 1 and 4, locomotor activity was measured during the pre-exposure injections (2–3 days apart, a total of 4 injections), while remaining injections were given in the home cage. * $p < 0.05$, ** $p < 0.01$, significant differences compared to saline pre-exposed animals as revealed by post-hoc Tukey comparisons following two-way repeated measure ANOVA. Numbers in parentheses indicate n/group.

4. Discussion

Following our previous results that repeated systemic administration of MPA produces locomotor sensitization similar to amphetamine in a dose-dependent manner (Yoon et al., 2016), we further showed in the present study that chronic MPA with the same experimental scheme resulting in locomotor sensitization also produces increase of spine density in the NAcc core. This is the first demonstration, to our knowledge, to indicate that MPA has an ability to induce structural plasticity in the NAcc with a similar appearance of dendritic spines as

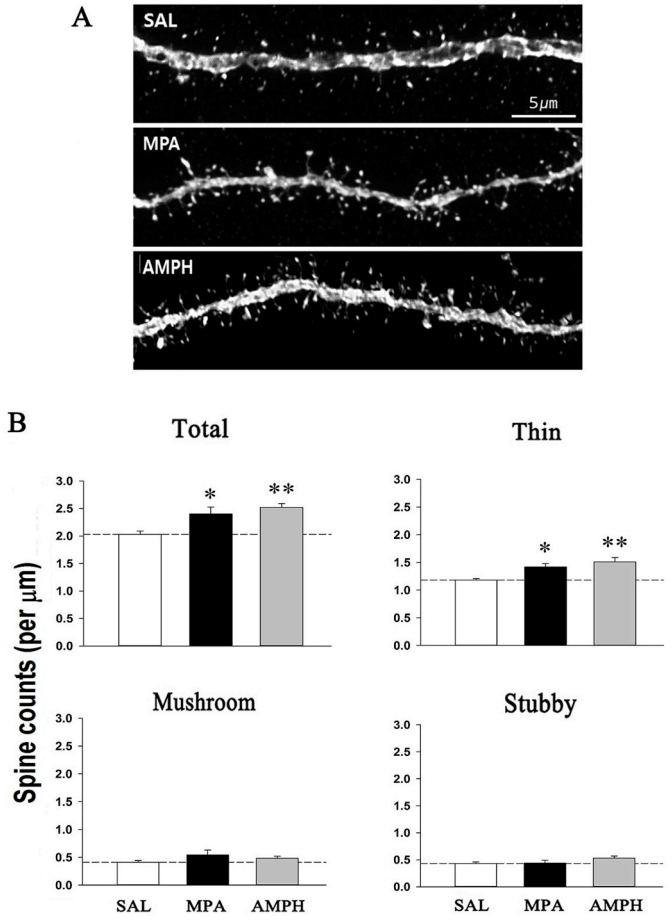

Fig. 2. Chronic injection of MPA or AMPH produces increases of total and thin spine densities in the NAcc core. (A) Representative high resolution images of a dendritic segment from each group. Images were obtained at magnification of 63× and with additional magnification of zoom 2.5 from ZEN software. (B) Significant increases were observed for the densities of total and thin spines in the NAcc, in both MPA (26 neurons from 5 animals) and AMPH (24 neurons from 5 animals) compared to saline pre-exposed rats (26 neurons from 5 animals). Data are expressed as actual values in spine density per μm. $^{*}p < 0.05$, $^{**}p < 0.01$, significant differences compared to saline pre-exposed animals as revealed by post-hoc Tukey comparisons following one-way ANOVA.

AMPH does in this site.

As locomotor sensitization, once developed, persists for a long period of time, it is considered as a specialized form of drug-induced long-term memory (Anagnostaras et al., 2002). In relation to this, it has been shown that locomotor sensitization induced by psychomotor stimulants is associated with the increase of dendritic spine densities in the NAcc (Robinson and Kolb, 1999; Li et al., 2003, 2004). Similarly, in the present study, we found that MPA, a structural analog to meth-AMPH, increases dendritic spine densities in this site when we applied the same experimental scheme previously shown to produce locomotor sensitization (Yoon et al., 2016). With our lack of concrete knowledge about functional roles for spine density and variability of spine subtypes, it is hard to draw any causal linkage between structural plasticity and addictive behavior (Russo et al., 2010). However, as previously shown for its role in the formation and the maintenance of enduring memory (Xu et al., 2009; Yang et al., 2009), it is certain that structural plasticity of dendritic spines somehow contributes to long-term changes of behavior including addiction (Russo et al., 2010). Similarly, there is open possibility that the changes of dendritic spine density after chronic exposure to MPA, or even to AMPH as well, may reflect some critical changes of neurophysiological and neurochemical properties of MSN cells in the NAcc, eventually leading to mediate addictive behaviors (i.e., locomotor sensitization in the present case) (Robinson and Kolb, 2004; Brown et al., 2011; Wang et al., 2013). Although there have been some reports showing that cocaine-induced morphological changes of dendritic spines in the NAcc, especially in the shell, could be dissociable from changes in addictive behavior (Pulipparacharuvil et al., 2008; Anderson et al., 2017), it still remains undetermined how possibly structural plasticity of spines contributes to the development of addictive behaviors. Considering development of addiction process consists of multiple stages, spine plasticity may differentially contribute according to the stage, as shown in one study, for example, that spine changes are dynamically altered in the NAcc core over different withdrawal periods from cocaine self-administration (Christian et al., 2017). As an alternative, spine plasticity may play a role as a compensatory mechanism to limit long-lasting addictive behaviors (Pulipparacharuvil et al., 2008).

Dendritic spines are the principal sites of excitatory input in the brain and commonly sub-typed into thin, stubby, and mushroom based on their different sizes and morphological features (Tada and Sheng, 2006; Rodriguez et al., 2008; Chidambaram et al., 2019). It is generally thought that thin spines are more flexible than other spine sub-types and more ready for plastic change in response to a variety of synaptic inputs (Bourne and Harris, 2007; Chidambaram et al., 2019). Thus, the increase of thin spines may provide the neuronal cell with more potential for subsequent plasticity. Interestingly, in the present study with MPA, the increase of total spine density in the NAcc core was mostly contributed by the increase of thin spines, suggesting that chronic MPA may induce production of thin spines to provide NAcc MSN cells with more potential for subsequent plasticity leading to addictive behaviors.

Different from thin spines, we did not observe any significant changes for mushroom and stubby spine densities in MPA as well as AMPH pre-exposed rats. Interestingly, it was recently shown that cell surface expression of AMPA receptors was not detected, while its phosphorylation was increased, by sensitizing exposure to AMPH in the rat NAcc (Wang et al., 2017). Because the head size of spine correlates well with the synaptic AMPA receptor expression levels (Matsuzaki et al., 2001; Kasai et al., 2003), these results indicate that no significant changes observed for mushroom and stubby spine densities in the NAcc after sensitizing exposure to MPA as well as AMPH in our present results are well correlated with biochemical findings in this site.

Repeated exposure to psychomotor stimulants is known to eventually lead to potentiate excitatory glutamatergic neurotransmission in the NAcc, which is thought to contribute to incentive motivational properties of drugs (Russo et al., 2010; Wolf and Ferrario, 2010; Brown et al., 2011; Wang et al., 2013, 2017). In our present study, we did not measure spine counts according to different stages of addictive process, so we don't know how they might be differently changed depending on the conditions (i.e., early versus late withdrawal period, during drug challenge, or after challenge, etc). Also, any accompanied molecular changes to provide spine's actual role in addictive behaviors completely remain in the future to be explored.

In conclusion, the present findings indicate that chronic MPA has an ability to provoke adaptive neuronal changes for total spine (especially thin spine) densities in the NAcc core in a similar way to AMPH, by which it may contribute to the expression of locomotor sensitization.

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