J Vet Sci. 2024 Sep;25(5):e63
https://doi.org/10.4142/jvs.23325
pISSN 1229-845X·eISSN 1976-555X

Journal of Veterinary Science

Research Report
Biotechnology

Check for updates

Different development patterns of reward behaviors induced by ketamine and JWH-018 in striatal GAD67 knockdown mice

Sun Mi Gu [1,†], **Eunchong Hong** [2,†], **Sowoon Seo** [1], **Sanghyeon Kim** [3], **Seong Shoon Yoon** [4], **Hye Jin Cha** [5,*], **Jaesuk Yun** [1,*]

[1]College of Pharmacy, Chungbuk National University, Cheongju 28160, Korea
[2]Non-Clinical Center, Osong Medical Innovation Foundation, Cheongju 28160, Korea
[3]Stanley Brain Research Laboratory, Stanley Medical Research Institute, Rockville, MD 20850, USA
[4]College of Korean Medicine, Daegu Haany University, Daegu 42158, Korea
[5]College of Veterinary Medicine, Gyeongsang National University, Jinju 52828, Korea

OPEN ACCESS

Received: Dec 28, 2023
Revised: Jun 25, 2024
Accepted: Jul 15, 2024
Published online: Aug 12, 2024

***Corresponding authors:**
Hye Jin Cha
College of Veterinary Medicine, Gyeongsang National University, 501 Jinju-daero, Jinju 52828, Korea.
Email: chahj@gnu.ac.kr
https://orcid.org/0009-0002-9523-0759

Jaesuk Yun
College of Pharmacy, Chungbuk National University, 194-31 Osongsaemgmyeong 1-ro, Osong-eup, Heungdeok-gu, Cheongju 28160, Korea.
Email: jyun@chungbuk.ac.kr
https://orcid.org/0000-0002-3093-8919

[†]Sun Mi Gu and Eunchong Hong equally contributed to this work.

ABSTRACT

Importance: Glutamic acid decarboxylase 67 (GAD67) is a gamma-aminobutyric acid (GABA) synthesis enzyme associated with the function of other neurotransmitter receptors, such as the N-methyl-D-aspartate (NMDA) receptor and cannabinoid receptor 1. However, the role of GAD67 in the development of different abused drug-induced reward behaviors remains unknown. In order to elucidate the mechanisms of substance use disorder, it is crucial to study changes in biomarkers within the brain's reward circuit induced by drug use.
Objective: The study was designed to examine the effects of the downregulation of GAD67 expression in the dorsal striatum on reward behavior development.
Methods: We evaluated the effects of GAD67 knockdown on depression-like behavior and anxiety using the forced swim test and elevated plus maze test in a mouse model. We further determined the effects of GAD67 knockdown on ketamine- and JWH-018-induced conditioned place preference (CPP).
Results: Knockdown of GAD67 in the dorsal striatum of mice increased depression-like behavior, but it decreased anxiety. Moreover, the CPP score on the NMDA receptor antagonist ketamine was increased by GAD67 knockdown, whereas the administration of JWH-018, a cannabinoid receptor agonist, did not affect the CPP score in the GAD67 knockdown mice group compared with the control group.
Conclusions and Relevance: These results suggest that striatal GAD67 reduces GABAergic neuronal activity and may cause ketamine-induced NMDA receptor inhibition. Consequently, GAD67 downregulation induces vulnerability to the drug reward behavior of ketamine.

Keywords: GAD67; gamma-aminobutyric acid; ketamine; JWH-018; conditioned place preference

INTRODUCTION

Drug abuse induces reward behaviors in human and animal models [1]. Dopamine released by the brain reward system plays a primary role in drug addiction. However,

ORCID iDs

Sun Mi Gu
https://orcid.org/0000-0002-7907-0487
Eunchong Hong
https://orcid.org/0009-0007-4882-6492
Sowoon Seo
https://orcid.org/0009-0006-8239-9570
Sanghyeon Kim
https://orcid.org/0009-0003-8823-3035
Seong Shoon Yoon
https://orcid.org/0000-0002-8182-1024
Hye Jin Cha
https://orcid.org/0009-0002-9523-0759
Jaesuk Yun
https://orcid.org/0000-0002-3093-8919

Author Contributions

Conceptualization: Cha HJ, Yun J; Data curation: Gu SM, Hong E; Formal Analysis: Gu SM, Hong E, Seo S, Kim S, Yoon SS; Funding acquisition: Cha HJ, Yun J; Investigation: Hong E, Seo S, Kim S, Yoon SS; Methodology: Kim S, Yoon SS; Project administration: Cha HJ, Yun J; Resources: Kim S, Yoon SS, Cha HJ, Yun J; Supervision: Cha HJ, Yun J; Visualization: Gu SM, Hong E, Seo S; Writing – original draft: Gu SM, Hong E; Writing – review & editing: Cha HJ, Yun J.

Conflict of Interest

The authors declare no conflicts of interest.

Funding

This work was supported by the Ministry of Food and Drug Safety (20182MFDS425, 22214MFDS252, 23212MFDS217), the National Research Foundation of Korea (NRF) grant funded by the Korean Government (MSIT) (No. MRC, 2017R1A5A2015541), "Regional Innovation Strategy (RIS)" through the NRF funded by the Ministry of Education (MOE) (2021RIS-001), the 2023 grant from The Korean Society of Ginseng, Basic Science Research Program through the NRF funded by the Ministry of Education (NRF-2021R1I1A1A01058188), and the research grant of the Gyeongsang National University in 2022.

other neurotransmitter systems are also affected by different drugs [2]. Specifically, gamma-aminobutyric acid (GABA)ergic interneurons regulate brain reward circuits via the modulation of dopamine neuronal activity [3]. Polymorphisms in GABAergic genes are involved in drug dependence and its consequences [4]. In addition, psychostimulants, including cocaine, amphetamine, and methylphenidate, induce variable changes in GABA synthesis isoenzymes, which are involved in the development of drug dependence [5]. In particular, glutamic acid decarboxylase 67 (GAD67) is encoded by glutamate decarboxylase 1 (GAD1) and may be involved in vulnerability to drug dependence [6]. GABAergic neurons are a major cell population in the striatum of the brain reward system [7]. However, the function of striatal GABAergic neurons in reward behavior is not well understood. GAD67 is a rate-limiting enzyme for GABA synthesis and is associated with motor deficits and mental disorders, such as schizophrenia, depression, and bipolar [8].

GAD67 is also associated with the cannabinoid system. Silencing GAD67 in cannabinoid receptor 1 (CB1)-positive cells induces abnormal behaviors related to schizophrenia and substance abuse in mice [9]. The psychostimulant activity of cannabinoids is associated with GABAergic neurotransmission [10]. The synthetic cannabinoid dehydroxylcannabidiol restores the isoform function of the major GABA A receptor [11]. The long-term use of cannabinoids results in dissociation between trigeminal sensory neuron GABA A receptor regulation and cyclic adenosine monophosphate changes [12]. In addition, the synthetic cannabinoid JWH-210 regulates GAD67 and CB1 expression in the brain [13]. JWH-018 is a synthetic cannabinoid with a high CB1 binding affinity. It acts through CB1 as Δ^9-THC to produce similar effects and attenuate Δ^9-THC withdrawal but may be increasingly abused because of its relatively short duration of action and greater potency at CB1 [14]. However, it is currently unknown whether GAD67 regulates JWH-018-induced reward behaviors. In this study, we examined the effects of the downregulation of GAD67 expression in the dorsal striatum on reward behavior development induced by JWH-018 in a conditioned place preference (CPP) mouse model.

Ketamine is classified as a dissociative anesthetic drug that can be abused [15]. It is an N-methyl-D-aspartate (NMDA) receptor antagonist, and ketamine abuse is often associated with severe social problems [16]. Depression and anxiety are risk factors for drug abuse [17]. GAD67 deficiency reported in mice exhibited sensitization to NDMA receptor antagonists and MK-801-induced locomotor activity [18]. Ketamine administration may alter GABA turnover and modulate NMDA receptor activity [19]. Ketamine increases the release of dopamine by blocking the NMDA receptors of GABA neurons and inhibiting the inhibition of dopaminergic neurons [20]. Therefore, we also aimed to evaluate the effects of GABAergic neurotransmission on NMDA receptor antagonist-induced psychological behaviors. Accordingly, the possible role of GAD67 in ketamine-induced CPP was investigated.

METHODS

Animals

C57BL/6 mice (male, 8–11 weeks old, 20–25 g) were purchased from Daehan Bio Link (Korea). The animals were maintained at 4–5 per cage under standard conditions (23 ± 2°C, 50 ± 5% humidity) with a controlled 12-h light/dark cycle. Drinking water and rodent chow were provided *ad libitum*. All experiments were performed in accordance with the Guidelines for the Care and Use of Animals (Animal Care Committee of Chungbuk National University, Korea [CBNUR-1453-20]).

Reagent

Synthetic cannabinoid JWH-018 was purchased from Cayman Chemical (USA). Ketamine was obtained from Yuhan Corporation (Korea). JWH-018 was administered at concentrations of 0.025 and 0.05 mg/kg via intraperitoneal (i.p.) injection. Ketamine was administered at concentrations of 5 and 10 mg/kg via i.p. injection.

Human RNA-sequencing

The RNA-sequencing (RNA-seq) data used in our previous study were reanalyzed in this study [21]. RNA samples were extracted from the NAcc of 79 individuals from the Array Collection (AC). RNA-seq experiment was performed by Psomagen Co. (USA). Briefly, cDNA libraries were prepared from 0.5 μg of RNA per sample using Illumina TruSeq RNA Library kit (Illumina Inc., USA) following the manufacturer's instructions. Sequencing libraries were created and sequenced with Illumina platform. Base calling and demultiplexing was done using bcl2fastq (bcl2fastq-1.8.4, Illumina). Quality control of the raw FASTQ files, mapping the RNA-seq reads and quantifying the map ped reads were performed as previously described with some modifications [21]. FASTQ files were generated from the Stanley Neuropathology Consortium Integrative Database (SNCID; http://sncid.stanleyresearch.org) [22]. The entire procedure is illustrated in supporting information (**Fig. 1**) individuals with past drug use but not at the time of death (past users; n = 15); 2) individuals using drugs at the time of death (present users; n = 14); 3) individuals who never used drugs (non-users; n = 50). In RNA-seq, the Institutional Review Board (IRB) of the Uniformed Services University of the Health Sciences, Bethesda, MD granted ethical approval for the Stanley Brain Collection. As the collection period was between 1998 and 2004 and the human participants deceased, the IRB determined that approval was not required as all specimens were de-identified and simply numbered. Consent to donate the specimens was obtained from next-of-kin and witnessed by two people who signed a form verifying the fact. Subsequently, the next-of-kin was contacted and interviewed to obtain further information about the deceased.

Knockdown of glutamic acid decarboxylase 67 (GAD67) expression in the mouse striatum using siRNA

Hair was removed from the surgical site following the anesthetization of the mouse with a 1.2% avertin solution. The incision site on the scalp was disinfected with 70% alcohol and cut. Bregma and lambda were identified, and the dorsal-ventral (DV) coordinates of the two locations were within ± 0.2 mm. A needle was moved to the bregma branch to designate its location. Thereafter, the needle was moved from the bregma coordinate to the coordinate corresponding to the X-axis +0.5 and the Y-axis +2.0. The needle of a Hamilton syringe was placed at 3.5 mm depth along the Z-axis, and 300 pmol scramble RNA (scrRNA) or GAD67 siRNA (Nos. 1360538, 1360539, and 1360540; Bioneer, Korea) was administered within a 1 μL delivery solution (jetSI, Polyplus Transfection, USA). The flow rate was 1 μL/min. One minute after the administration, the needle was slowly removed. The incision was sutured using Biobond. After allowing a 7-day period for recovery and siRNA activation, behavioral tests were performed or the subjects were euthanized, and the striatum of the brain was collected and stored at −80°C.

Knockdown of glutamic acid decarboxylase 67 (GAD67) expression in the mouse striatum using CRISPR/Cas9

Mice were injected with control or GAD67 CRISPR/Cas9 gRNA vector (eGFP tagged, Macrogen, Korea) to induce GAD67 knockdown. Following the anesthetization of the mouse with 1.2% avertin solution, hair was removed from the surgical site. The scalp incision site

Journal of Veterinary Science

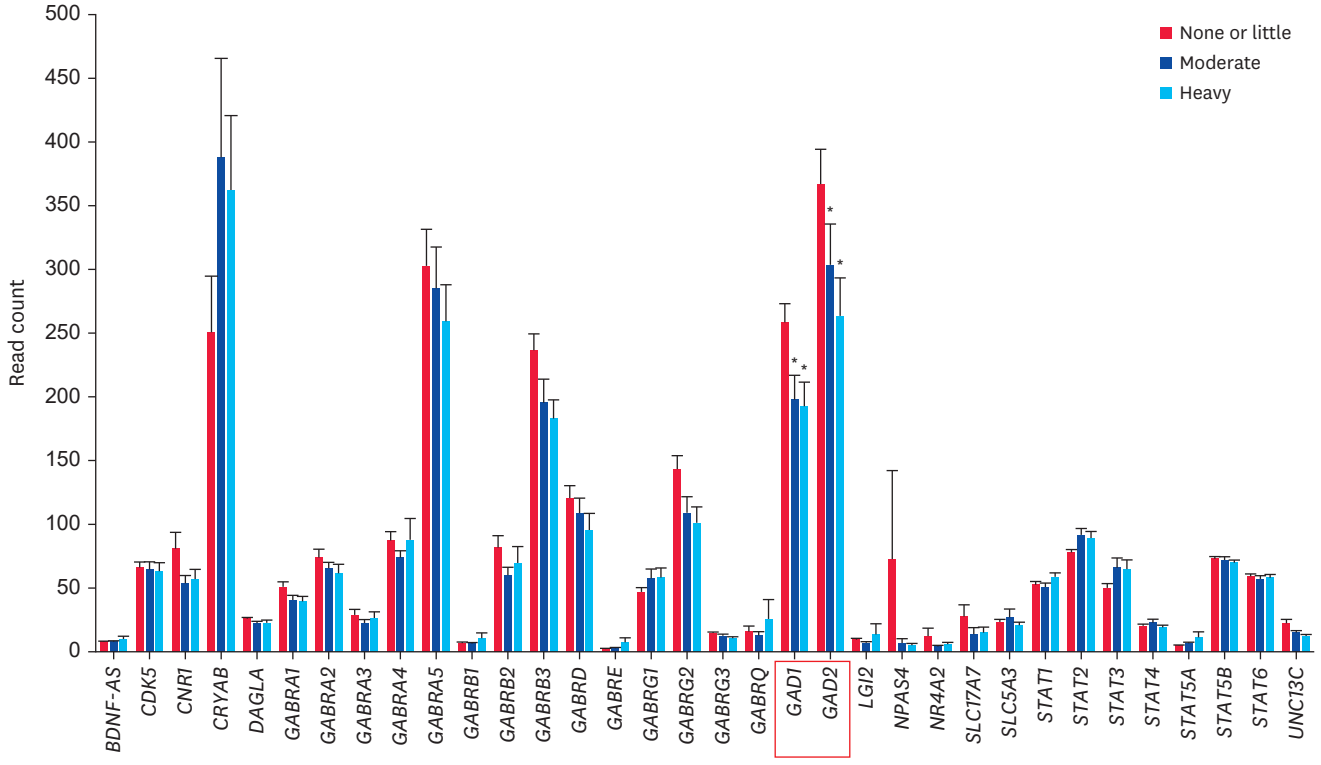

Fig. 1. Gene expression according to drug usage in the human nucleus accumbens. The mean RNA-sequencing read counts of *GAD1* gene in each severity rating. Data are expressed as the mean ± standard error (n = 8, 15, and 14 for the none or little, moderate, and heavy groups, respectively) and were analyzed using a two-way ANOVA (*$p < 0.05$ vs. none or little group).
BDNF-AS, BDNF antisense RNA; *CDK5*, cyclin dependent kinase 5; *CNR1*, cannabinoid receptor 1; *CRYAB*, crystallin alpha B; *DAGLA*, diacylglycerol lipase alpha; *GABRA1*, gamma-aminobutyric acid type A receptor subunit alpha1; *GABRA2*, gamma-aminobutyric acid type A receptor subunit alpha2; *GABRA3*, gamma-aminobutyric acid type A receptor subunit alpha3; *GABRA4*, gamma-aminobutyric acid type A receptor subunit alpha4; *GABRA5*, gamma-aminobutyric acid type A receptor subunit alpha5; *GABRB1*, gamma-aminobutyric acid type A receptor subunit beta1; *GABRB2*, gamma-aminobutyric acid type A receptor subunit beta2; *GABRB3*, gamma-aminobutyric acid type A receptor subunit beta3; *GABRD*, gamma-aminobutyric acid type A receptor subunit delta; *GABRE*, gamma-aminobutyric acid type A receptor subunit epsilon; *GABRG1*, gamma-aminobutyric acid type A receptor subunit gamma1; *GABRG2*, gamma-aminobutyric acid type A receptor subunit gamma2; *GABRG3*, gamma-aminobutyric acid type A receptor subunit gamma3; *GABRQ*, gamma-aminobutyric acid type A receptor subunit theta; *GAD1*, glutamate decarboxylase 1; *GAD2*, glutamate decarboxylase 2; *LGI2*, leucine rich repeat LGI family member 2; *NPAS4*, neuronal PAS domain protein 4; *NR4A2*, nuclear receptor subfamily 4 group A member 2; *SLC17A7*, solute carrier family 17 member 7; *SLC5A3*, solute carrier family 5 member 3; *STAT1*, signal transducer and activator of transcription 1; *STAT2*, signal transducer and activator of transcription 2; *STAT3*, signal transducer and activator of transcription 3; *STAT4*, signal transducer and activator of transcription 4; *STAT5A*, signal transducer and activator of transcription 5A; *STAT5B*, signal transducer and activator of transcription 5B; *STAT6*, signal transducer and activator of transcription 6; *UNC13C*, unc-13 homolog C.

was disinfected using 70% alcohol. The bregma and lambda were located, and we ensured that the DV coordinates of the two locations were within ± 0.2 mm. A needle was moved to the bregma branch and designated. Thereafter, the needle was moved from the bregma coordinate to the coordinate corresponding to the X-axis +0.5 and the Y-axis +2.0. The needle of the Hamilton syringe was placed at a depth of 3.5 mm along the Z-axis. A 0.4 μg control or GAD67 CRISPR/Cas9 gRNA vector was administered through a 1.5 μL delivery solution (in vivo-jetPEI, Polyplus Transfection). The flow rate was 1.5 μL/min. The needle was slowly removed 1 min after administration. The incision was sutured using Biobond. After waiting for 7 days for recovery and vector activity, a behavioral experiment or autopsy was conducted, whereby the brain was perfused with 4% paraformaldehyde (PFA), extracted, placed in 4% PFA, and stored at 4°C.

Western blotting

GAD67 siRNA or scrRNA was administered to the right brain striatum via stereotaxic injection, and a sample of the right brain striatum was extracted from the mouse on the fourth day.

https://doi.org/10.4142/jvs.23325

The reagent was based on 1× Tris-buffered saline with Tween (TBST). Lysis buffer (20 mM Tris-HCl, 250 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1% Triton X-100) was used to extract proteins from the striatum. Following quantification of the protein concentration using the Bradford reagent, a sample was produced using sterile distilled water and a 5× sample buffer. Protein (30 μg) was added to a 12% sodium dodecyl sulfate-polyacrylamide gel, electrophoresed, and transferred to the immobilon-polyvinylidene difluoride membrane (0.45 μm pore size, Immobilon-P PVDF, Millipore, USA). The membrane was blocked with 5% skim milk (Difco, USA). The primary antibodies, GAD67 (1:1,000, ab26116, Abcam, USA) and GAPDH (1:5,000, 2118S, Cell Signaling Technology, USA), were diluted with 5% skin milk and reacted for at least 12 h in a gintang culture medium at 4°C. The samples were washed thrice with TBST for 10 min. The secondary mouse (1:5,000, Sigma) and rabbit (1:10,000, Sigma) antibodies were diluted with 5% skin milk and reacted at room temperature (RT) for 1–2 h. They were then washed thrice with TBST for 10 min and detected using the Fusion Solo S (Vilber Lourmat, France) machine and software with ECL solutions. The confirmed protein was quantified using the ImageJ software (Wayne Rasband, National Institutes of Health, USA).

Immunohistochemistry

Mice were euthanized by CO_2 inhalation and perfused with phosphate-buffered saline (PBS, pH 7.4) containing heparin and 4% PFA in PBS (pH 7.4) at the end of the behavior tests. The brains of the mice were processed and cut into brain sections (10 μm) following the methods described in a previous study. Before staining, the brain sections were air-dried for 3 h. After two 10-min washes in PBS (pH 7.4), the brain sections were incubated at 60°C in citrate buffer (10 mM citric acid, pH 7.4) for 30 min and then incubated overnight with mouse anti-GAD67 (1:300, Sigma Aldrich, USA) primary antibody at 4°C. Next, the brain sections were incubated with a secondary antibody conjugated to biotinylated goat anti-mouse IgG-horseradish peroxidase (1:500, Santa Cruz, USA) at RT. The brain sections were evaluated using a light microscope (Axio Imager.A2, Carl Zeiss, Germany, ×200).

Locomotor activity test

To investigate the effects of GAD67 knockdown on mouse movement, mice were stereotaxically injected with scrRNA or GAD67 siRNA, as described above. After their adaptation to the test cage for 60 min, locomotor activity was measured at 5-min intervals for 120 min using an automatic tracking system.

EPM test

The control or GAD67 gRNA vector was administered to the right brain striatum, and C57BL/6 mice were used after a 7-day rest period. An instrument consisting of a center (10 cm × 10 cm) with two open arms (35 cm × 10 cm) and two closed arms (35 cm × 10 cm) met each other at a distance of 50 cm from the floor was used. The illumination of each zone was maintained at 170 lux throughout the experiment. One mouse was placed at the center of the elevated plus maze (EPM) instrument, and its movement to each zone for 4 min was recorded using the SMART-LD program (Panlab, Spain).

Forced swim test (FST)

The control or GAD67 gRNA vector was administered to the right brain striatum, and C57BL/6 mice were used after a 7-day rest period, followed by the EPM tests. A cylinder with a diameter of 20 cm and a height of 30 cm was used. During the experiment, one mouse was placed in the cylinder, and movements were recorded using a video-tracking program (SMART-LC, Panlab) for 5 min.

Conditioned place preference

The control or GAD67 gRNA vector was administered to the right brain striatum, and the mice were allowed to rest for 7 days. As a negative control group, saline was administered to the abdominal cavity at a rate of 1 mL per 100 g weight. The test used consisted of three stages: 1) habituation and pre-recording, 2) conditioning, and 3) post-recording. During the habituation phase, the mouse had access to both spaces for 15 min for 3 consecutive days. The time spent in each compartment was recorded for 15 min from day 4. The mouse was assigned to the group according to the pre-conditioning phase; in particular, the least preferred side was designated as the drug administration space. During the conditioning phase, the guillotine door was closed. Ketamine (5 mg/kg, 10 mg/kg), JWH-018 (0.025, 0.05 mg/kg), and saline (1 mg/kg) conditioning occurred for 30 min via the abdominal cavity. Every other day, the mice were injected with saline and conditioned in a space opposite to the drug. Immediately after the last conditioning date, a recording was performed in which the mouse had access to both spaces in the pre-conditioning stage without taking any medication.

Data analysis

Data represent the mean ± standard error. The data were analyzed using Student's *t*-test and two-way analysis of variance, followed by a Holm-Šídák post-hoc *t*-test. All analyses were performed using the SigmaPlot 14 software (Systat Software, USA).

RESULTS

The expression of glutamate decarboxylase 1 in the human nucleus accumbens was reduced in the heavy group

RNA-seq data used in a previous study were reanalyzed, and RNA-seq analysis was performed on samples from the nucleus accumbens of drug users. The sample groups were divided into the "None or little," "Moderate," and "Heavy" groups. "None or little" indicates no history or little of drug use, "Moderate" indicates a history of moderate drug use, and "Heavy" denotes a history of heavy drug use in the past. Gene expression of *GAD1* was lower in "Heavy" than in "None or little"(**Fig. 1**).

The expression of GAD67 in the striatum was reduced by GAD67 siRNA and GAD67 CRISPR/Cas9 gRNA vector

A stereotaxic injection of 300 pmol/μL scrRNA or GAD67 siRNA was administered to the right brain striatum at a rate of 1 μL/min. Consequently, GAD67 expression was significantly lower after GAD67 siRNA administration compared with that after scrRNA administration (**Fig. 2A**). The stereotaxic injection of GAD67 CRISPR/Cas9 gRNA vector reduced GAD67 expression in the striatum (**Fig. 2B and C**).

Locomotor activity results had no change in GAD67 siRNA treatment

Locomotor activity test was performed without any other drug injections. Compared with scrRNA mice, locomotor activity test results had no effect on GAD67 knockdown mice (**Fig. 3**).

GAD67 KD mice showed increased anxiety, with their depression significantly unchanged

The FST is an antidepressant efficacy testing method that allows a relatively narrow cylinder to be filled with water at a height outside the reach of the floor, forcing the mouse to swim. The immobility of the control vector group tended to increase, but was not statistically

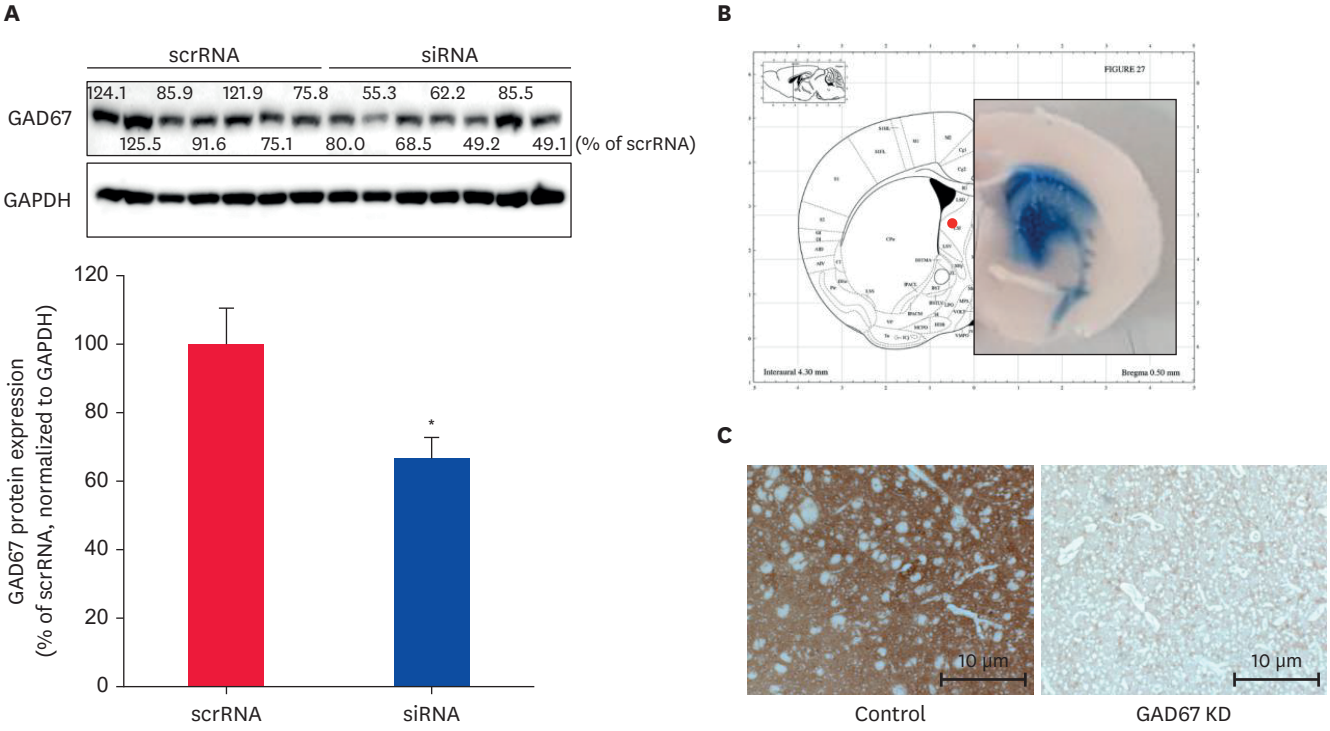

Fig. 2. Confirmations of GAD67 protein expression and working of the CRISPR/Cas9 system in mice striatum. (A) Mice were stereotaxically treated with either a negative control (scrRNA) or GAD67 siRNA in the right striatum. The protein expression of GAD67 was confirmed by western blotting. Expression of each protein was normalized to the relative amplification of GAPDH. Data are expressed as the mean ± standard error (n = 7 for each group) and were analyzed using a Student's *t*-test (*$p < 0.05$ vs. scrRNA group). (B) Mice were stereotaxically injected with the control or GAD67 gRNA CRISPR/Cas9 vector. (C) Immunohistochemical staining of GAD67 in the mice striatum sections (10 μm thick). Original magnification is ×200, scale bar = 10 μm.
GAD67, glutamic acid decarboxylase 67; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; scrRNA, scramble RNA.

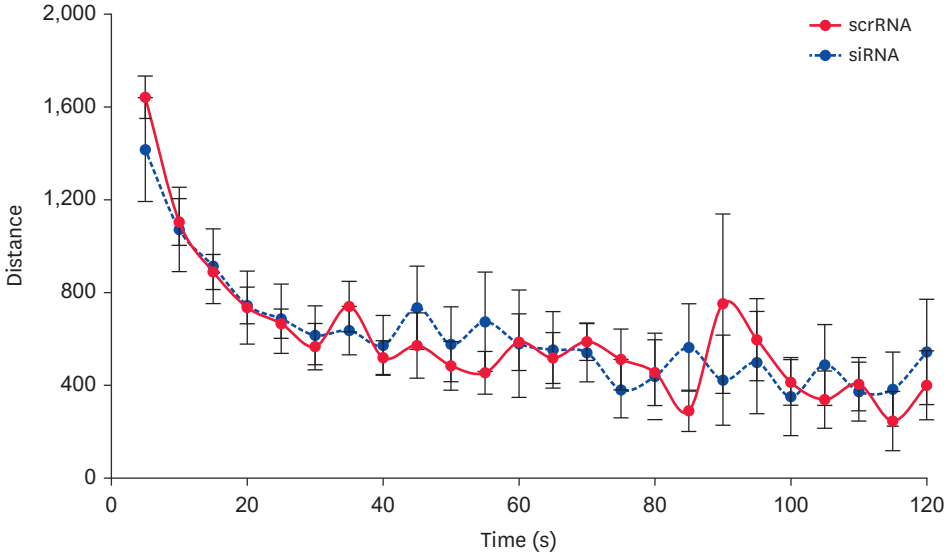

Fig. 3. Effects of GAD67 knockdown on locomotor activity test. Locomotor activity was measured for 120 min without any drug challenge. Data are expressed as the mean ± SE (n = 5–6 for each group) and were analyzed using a one-way RM ANOVA.
GAD67, glutamic acid decarboxylase 67; SE, standard error; RM ANOVA, repeated measures analysis of variance.

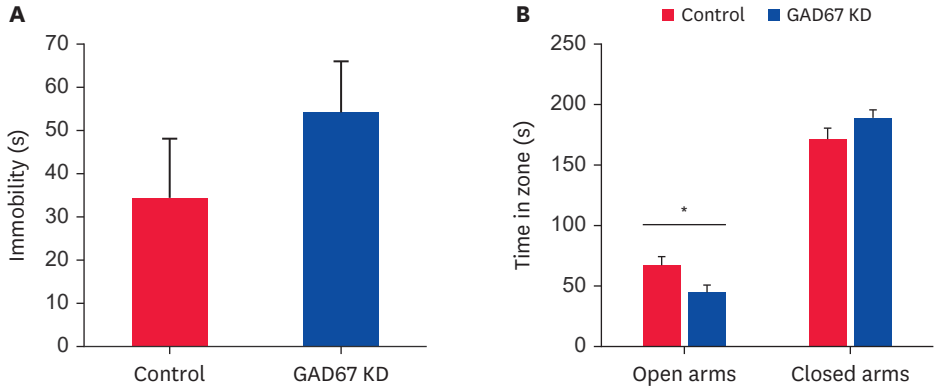

Fig. 4. Effects of GAD67 knockdown on the FST and EPM test. (A) The immobility of the control vector group during the FST was measured for 5 min. Data are expressed as the mean ± standard error (n = 9 for control group and 12 for GAD67 KD group, respectively) and were analyzed using a Student's *t*-test. (B) The time in each zone was measured in EPM for 4 min. Data are expressed as the mean ± standard error (n = 9 for control group and 12 for GAD67 KD group, respectively) and were analyzed using a Student's *t*-test ($^{*}p < 0.05$ vs. control).
GAD67, glutamic acid decarboxylase 67; FST, forced swim test; EPM, elevated plus maze.

significant compared to that of the vector containing GAD67 gRNA using the CRISPR/Cas9 system administered to the mouse striatum (**Fig. 4A**). The mouse explored new places and was conflicted between the desire to enter open or closed areas to avoid anxiety regarding unfamiliarity. The EPM tests were conducted to determine which needs were greater. Comparison of the control vector administered to the mouse striatum with the vector containing GAD67 gRNA using the CRISPR/Cas9 system revealed no significant difference in the open, closed, and central regions. However, the GAD67 knockdown group had significantly lower time in the open region compared with the control group (**Fig. 4B**).

Ketamine increased reward behavior in GAD67 knockdown mice

The CPP score increased significantly in C57BL/6 mice treated with ketamine (10 mg/kg). However, the number of mice treated with 5 mg/kg ketamine also increased, but this increase was not significant (**Fig. 5A**). A comparison of CPPs with 5 mg/kg ketamine was performed in the GAD67 knockdown group of mice. A significant increase in CPP with 5 mg/kg ketamine in GAD67 knockdown mice was observed (**Fig. 5B**).

The reward behavior for JWH-018 was unchanged in GAD67 knockdown mice

Within mice administered either the control or CRISPR/Cas9 GAD67 gRNA vector to the striatum, CPPs for JWH-018 0.025 and 0.05 mg/kg tended to increase in a concentration-dependent manner, but they were not significant. Furthermore, GAD67 knockdown did not affect the CPP for 0.5 mg/kg JWH-018 0.025 (**Fig. 6**).

DISCUSSION

Several neurotransmitter systems are associated with the development of reward behaviors. CPPs are used for reward behavior animal models based on the Pavlovian conditioning paradigm [23]. GABAergic, dopaminergic, glutamatergic, and serotonergic neurotransmission in the striatum is associated with CPP development [24,25]. In this study, we demonstrated that an *in vivo* the CRISPR/Cas9 GAD67 gRNA vector reduced GAD67 expression in the dorsal striatum of mice. GAD67 knockdown mice had lower open arm time in the EPM test compared with control mice. However, depression-like behavior

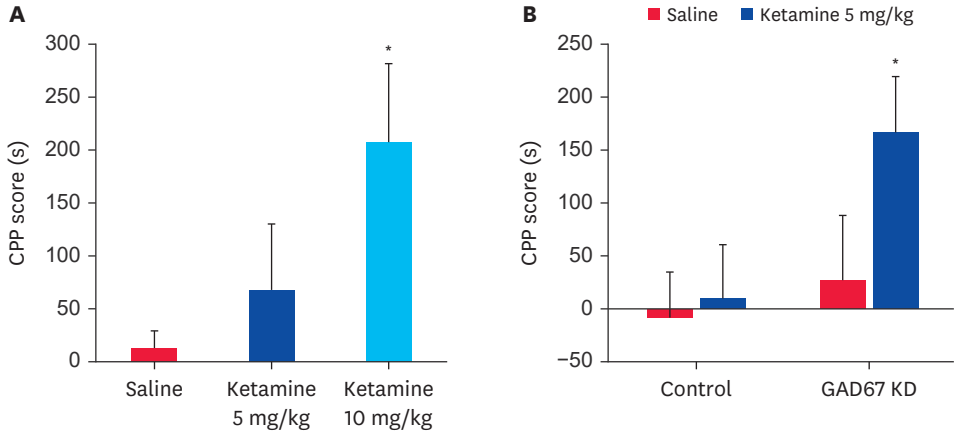

Fig. 5. Effects of GAD67 knockdown on ketamine-induced CPP. (A) CPP test performed using saline or ketamine (5 mg/kg and 10 mg/kg, i.p.) in C57BL/6 mice. Data are expressed as the mean ± standard error (n = 13, 7, and 6 for saline, ketamine 5 mg/kg, and ketamine 10 mg/kg, respectively) and were analyzed using a one-way ANOVA ($F[2,23] = 4.829$, $p = 0.017$) followed by a Holm-Šídák post-hoc *t*-test. ($^*p < 0.05$ vs. 10 mg/kg saline group). (B) Mice were stereotaxically treated once with either a negative control or CRISPR/Cas9 GAD67 gRNA vector in the right striatum. Thereafter, the CPP test was performed, as described in the Methods section, using saline or ketamine (5 mg/kg, i.p.) 7 days after striatal injection. Data are expressed as the mean ± standard error (n = 7, 9, 11, and 9 for control & saline, control & ketamine 5 mg/kg, GAD67 KD & saline, and GAD67 KD & ketamine 5 mg/kg, respectively) and were analyzed using a two-way ANOVA (GAD67 condition: $F[1,32] = 1.993$, $p = 0.168$; CPP drug condition: $F[1,32] = 2.958$, $p = 0.095$; interaction: $F[1,32] = 1.182$, $p = 0.285$) followed by a Holm-Šídák post-hoc *t*-test ($^*p < 0.05$ vs. GAD67 KD saline group).
GAD67, glutamic acid decarboxylase 67; CPP, conditioned place preference; i.p., intraperitoneal; ANOVA, analysis of variance; KD, knockdown.

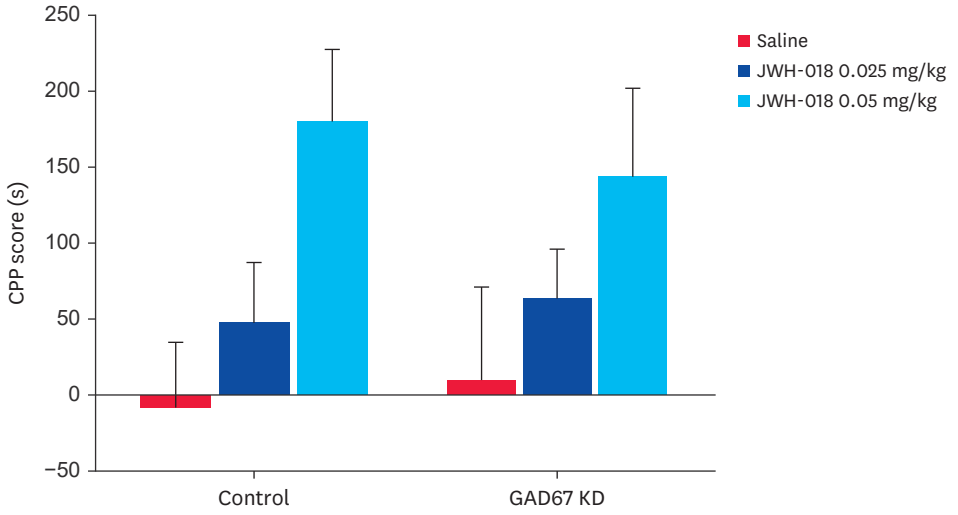

Fig. 6. Effect of GAD67 knockdown on JWH-018-induced CPP. Mice were stereotaxically treated once with either a negative control or the CRISPR/Cas9 GAD67 gRNA vector in the right striatum. Thereafter, the CPP test was performed, as described in the Methods section, using saline or JWH-018 (0.025 or 0.05 mg/kg, i.p.) 7 days after striatal injection. Data are expressed as the mean ± standard error (n = 7, 7, 5, 1, 8, and 9 for control & saline, control & JWH-018 0.025 mg/kg, control & JWH-018 0.05 mg/kg, GAD67 KD & saline, GAD67 KD & JWH-018 0.025 mg/kg, and GAD67 KD & JWH-018 0.05 mg/kg, respectively) and were analyzed using a two-way ANOVA (GAD67 condition: $F[1,41] = 0.00026$, $p = 0.987$; CPP drug condition: $F[1,41] = 4.361$, $p = 0.019$; interaction: $F[1,41] = 0.149$, $p = 0.862$) followed by a Holm-Šídák post-hoc *t*-test.
GAD67, glutamic acid decarboxylase 67; CPP, conditioned place preference; i.p., intraperitoneal.

in knockdown mice was not observed in the FST. These results may be associated with the altered GABAergic modulation on dopaminergic neuronal function in the striatum [26]. Striatal GABAergic neuronal function is also important for the regulation of normal limb movements in rodent models [27]. However, in our study, the knockdown of GAD67 did

not induce abnormal behavior in the climbing behavior test (unpublished data), which was used for the striatal dopamine receptor functional test model [28]. Therefore, we assume that downregulation of GAD67 does not alter dopamine receptor function and activity. GABAergic output from medium spiny neurons (MSNs) in the striatum consists of brain reward circuits projecting into the midbrain area [29]. The GABAergic pathway from the MSNs of the striatum to the midbrain dopaminergic neuronal pathway has been associated with reward stimulation via interneuron regulation in the VTA. In this study, downregulated GAD67 mice had higher CPP score induced by ketamine administration compared with control mice. Moreover, downregulation of *GAD1* was also observed in striatal samples from individuals with a history of heavy drug use. *GAD1* encodes GAD67 and is known to be involved in the regulation of basal GABA levels in mammals [30]. The results of the present study could support the previous findings suggesting that *GAD1* might be a risk factor for drug dependence [31]. Therefore, the significance of the present study lies in demonstrating the association of *GAD1* (or GAD67) with drug dependence in both mice and humans.

Repeated administration of NMDA receptor antagonists, namely, ketamine, a ketamine-derivative, and methoxetamine, induced CPP in mice [32]. Abuse potential of NMDA receptor antagonists is associated with stimulation of the dopamine reward system [33].

Methoxetamine and ketamine can inhibit dopamine reuptake, which is associated with drug reward behaviors [32]. Furthermore, an *in vitro* study demonstrated that ketamine possessed dopamine transporter antagonistic activity [34]. Ketamine treatment also reduces GAD67 expression, especially in parvalbumin-positive interneurons, suggesting that NMDA receptor signaling may be correlated with GAD67 function [35]. These results indicate that downregulation of GAD67 in the striatum augments ketamine-induced dopaminergic neurotransmission via disinhibition of interneurons.

In contrast to ketamine, CPP development induced by JWH-018 in GAD67 knockdown mice was not higher than that in the control mice. JWH-018 is a synthetic cannabinoid with high affinity to CB1 [36]. Activation of CB1 on the presynaptic terminal of GABAergic interneurons may induce dopaminergic neuronal activation and reward behaviors via VTA disinhibition [37]. Cannabinoids may increase the release and synthesis of dopamine via cell firing [38]. However, dopamine reuptake inhibition is not associated with the effects of JWH-018 on the dopamine system [10]. The mechanism of action of JWH-018 may explain the different patterns of CPP development in GAD67 knockdown mice.

JWH-018 treatment activates mitogen-activated protein kinase (MAPK) in CB1-expressed HEK293 cells [39]. However, GABAergic neuronal activation did not stimulate an MAPK cascade in mature cultured neurons. Furthermore, GAD67 deficiency induces a reduction in CB1 expression in mice [40]. Therefore, the mechanism of action of JWH-018 and reduced CB1 expression may explain the different patterns of CPP development in GAD67 knockdown mice.

REFERENCES

1. Cooper S, Robison AJ, Mazei-Robison MS. Reward circuitry in addiction. *Neurotherapeutics*. 2017;14(3):687-697. PUBMED | CROSSREF
2. Herman MA, Roberto M. The addicted brain: understanding the neurophysiological mechanisms of addictive disorders. *Front Integr Nuerosci*. 2015;9:18. PUBMED | CROSSREF

3. Vashchinkina E, Panhelainen A, Aitta-Aho T, Korpi ER. GABAA receptor drugs and neuronal plasticity in reward and aversion: focus on the ventral tegmental area. *Front Pharmacol*. 2014;5:256. PUBMED | CROSSREF
4. Nudmamud-Thanoi S, Veerasakul S, Thanoi S. Pharmacogenetics of drug dependence: Polymorphisms of genes involved in GABA neurotransmission. *Neurosci Lett*. 2020;726:134463. PUBMED | CROSSREF
5. Freese L, Muller EJ, Souza MF, Couto-Pereira NS, Tosca CF, Ferigolo M, et al. GABA system changes in methylphenidate sensitized female rats. *Behav Brain Res*. 2012;231(1):181-186. PUBMED | CROSSREF
6. Wu W, Zhu YS, Li SB. Polymorphisms in the glutamate decarboxylase 1 gene associated with heroin dependence. *Biochem Biophys Res Commun*. 2012;422(1):91-96. PUBMED | CROSSREF
7. Ade KK, Janssen MJ, Ortinski PI, Vicini S. Differential tonic GABA conductances in striatal medium spiny neurons. *J Neurosci*. 2008;28(5):1185-1197. PUBMED | CROSSREF
8. Heusner CL, Beutler LR, Houser CR, Palmiter RD. Deletion of GAD67 in dopamine receptor-1 expressing cells causes specific motor deficits. *Genesis*. 2008;46(7):357-367. PUBMED | CROSSREF
9. Brown JA, Horváth S, Garbett KA, Schmidt MJ, Everheart M, Gellért L, et al. The role of cannabinoid 1 receptor expressing interneurons in behavior. *Neurobiol Dis*. 2014;63:210-221. PUBMED | CROSSREF
10. Ossato A, Uccelli L, Bilel S, Canazza I, Di Domenico G, Pasquali M, et al. Psychostimulant effect of the synthetic cannabinoid JWH-018 and AKB48: behavioral, neurochemical, and dopamine transporter scan imaging studies in mice. *Front Psychiatry*. 2017;8:130. PUBMED | CROSSREF
11. Zou G, Xia J, Han Q, Liu D, Xiong W. The synthetic cannabinoid dehydroxylcannabidiol restores the function of a major $GABA_A$ receptor isoform in a cell model of hyperekplexia. *J Biol Chem*. 2020;295(1):138-145. PUBMED | CROSSREF
12. Celotto L, Eroli F, Nistri A, Vilotti S. Long-term application of cannabinoids leads to dissociation between changes in cAMP and modulation of $GABA_A$ receptors of mouse trigeminal sensory neurons. *Neurochem Int*. 2019;126:74-85. PUBMED | CROSSREF
13. Gu SM, Seo S, Park D, Kim S, Lamichhane S, Han KM, et al. Cannabinoid receptor type 1 regulates drug reward behavior via glutamate decarboxylase 67 transcription. *Int J Mol Sci*. 2021;22(19):10486. PUBMED | CROSSREF
14. Gatch MB, Forster MJ. Δ9-Tetrahydrocannabinol-like discriminative stimulus effects of compounds commonly found in K2/Spice. *Behav Pharmacol*. 2014;25(8):750-757. PUBMED | CROSSREF
15. Liu Y, Lin D, Wu B, Zhou W. Ketamine abuse potential and use disorder. *Brain Res Bull*. 2016;126(Pt 1):68-73. PUBMED | CROSSREF
16. Tang WK, Liang HJ, Lau CG, Tang A, Ungvari GS. Relationship between cognitive impairment and depressive symptoms in current ketamine users. *J Stud Alcohol Drugs*. 2013;74(3):460-468. PUBMED | CROSSREF
17. Chung EY, Cha HJ, Min HK, Yun J. Pharmacology and adverse effects of new psychoactive substances: synthetic cannabinoid receptor agonists. *Arch Pharm Res*. 2021;44(4):402-413. PUBMED | CROSSREF
18. Fujihara K, Miwa H, Kakizaki T, Kaneko R, Mikuni M, Tanahira C, et al. Glutamate decarboxylase 67 deficiency in a subset of GABAergic neurons induces schizophrenia-related phenotypes. *Neuropsychopharmacology*. 2015;40(10):2475-2486. PUBMED | CROSSREF
19. Silberbauer LR, Spurny B, Handschuh P, Klöbl M, Bednarik P, Reiter B, et al. Effect of ketamine on limbic GABA and glutamate: a human *in vivo* multivoxel magnetic resonance spectroscopy study. *Front Psychiatry*. 2020;11:549903. PUBMED | CROSSREF
20. Pham TH, Gardier AM. Fast-acting antidepressant activity of ketamine: highlights on brain serotonin, glutamate, and GABA neurotransmission in preclinical studies. *Pharmacol Ther*. 2019;199:58-90. PUBMED | CROSSREF
21. Kim S, Jo Y, Webster MJ, Lee D. Shared co-expression networks in frontal cortex of the normal aged brain and schizophrenia. *Schizophr Res*. 2019;204:253-261. PUBMED | CROSSREF
22. Kim S, Webster MJ. The Stanley Neuropathology Consortium Integrative Database (SNCID) for psychiatric disorders. *Neurosci Bull*. 2019;35(2):277-282. PUBMED | CROSSREF
23. Tzschentke TM. Conditioned place preference and aversion, In: Stolerman IP, Price LH, eds. *Encyclopedia of Psychopharmacology*. Springer; 2010: p1-7.
24. Chu J, Deyama S, Li X, Motono M, Otoda A, Saito A, et al. Role of $5\text{-}HT_{1A}$ receptor-mediated serotonergic transmission in the medial prefrontal cortex in acute restraint stress-induced augmentation of rewarding memory of cocaine in mice. *Neurosci Lett*. 2021;743:135555. PUBMED | CROSSREF
25. Weitz M, Khayat A, Yaka R. GABAergic projections to the ventral tegmental area govern cocaine-conditioned reward. *Addict Biol*. 2021;26(4):e13026. PUBMED | CROSSREF
26. Dong MX, Chen GH, Hu L. Dopaminergic system alteration in anxiety and compulsive disorders: a systematic review of neuroimaging studies. *Front Neurosci*. 2020;14:608520. PUBMED | CROSSREF

27. Zhang K, Chammas C, Soghomonian JJ. Loss of glutamic acid decarboxylase (Gad67) in striatal neurons expressing the Drdr1a dopamine receptor prevents L-DOPA-induced dyskinesia in 6-hydroxydopamine-lesioned mice. *Neuroscience*. 2015;303:586-594. PUBMED | CROSSREF

28. Bricker BA, Peprah K, Kang HJ, Ablordeppey SY. Evaluation of SYA16263 as a new potential antipsychotic agent without catalepsy. *Pharmacol Biochem Behav*. 2019;179:55-62. PUBMED | CROSSREF

29. Cox J, Witten IB. Striatal circuits for reward learning and decision-making. *Nat Rev Neurosci*. 2019;20(8):482-494. PUBMED | CROSSREF

30. Kuo PH, Kalsi G, Prescott CA, Hodgkinson CA, Goldman D, Alexander J, et al. Associations of glutamate decarboxylase genes with initial sensitivity and age-at-onset of alcohol dependence in the Irish Affected Sib Pair Study of Alcohol Dependence. *Drug Alcohol Depend*. 2009;101(1-2):80-87. PUBMED | CROSSREF

31. Levran O, Peles E, Randesi M, Correa da Rosa J, Ott J, Rotrosen J, et al. Glutamatergic and GABAergic susceptibility loci for heroin and cocaine addiction in subjects of African and European ancestry. *Prog Neuropsychopharmacol Biol Psychiatry*. 2016;64:118-123. PUBMED | CROSSREF

32. Botanas CJ, de la Peña JB, Dela Peña IJ, Tampus R, Yoon R, Kim HJ, et al. Methoxetamine, a ketamine derivative, produced conditioned place preference and was self-administered by rats: evidence of its abuse potential. *Pharmacol Biochem Behav*. 2015;133:31-36. PUBMED | CROSSREF

33. Marcus MM, Mathé JM, Nomikos GG, Svensson TH. Effects of competitive and non-competitive NMDA receptor antagonists on dopamine output in the shell and core subdivisions of the nucleus accumbens. *Neuropharmacology*. 2001;40(4):482-490. PUBMED | CROSSREF

34. Dedic N, Kühne C, Jakovcevski M, Hartmann J, Genewsky AJ, Gomes KS, et al. Chronic CRH depletion from GABAergic, long-range projection neurons in the extended amygdala reduces dopamine release and increases anxiety. *Nat Neurosci*. 2018;21(6):803-807. PUBMED | CROSSREF

35. Kinney JW, Davis CN, Tabarean I, Conti B, Bartfai T, Behrens MM. A specific role for NR2A-containing NMDA receptors in the maintenance of parvalbumin and GAD67 immunoreactivity in cultured interneurons. *J Neurosci*. 2006;26(5):1604-1615. PUBMED | CROSSREF

36. Walsh KB, Andersen HK. Molecular Pharmacology of Synthetic Cannabinoids: Delineating CB1 Receptor-Mediated Cell Signaling. *Int J Mol Sci*. 2020;21(17):6115. PUBMED | CROSSREF

37. Tepper JM, Koós T, Ibanez-Sandoval O, Tecuapetla F, Faust TW, Assous M. Heterogeneity and diversity of striatal GABAergic interneurons: update 2018. *Front Neuroanat*. 2018;12:91. PUBMED | CROSSREF

38. Bloomfield MA, Ashok AH, Volkow ND, Howes OD. The effects of Δ^9-tetrahydrocannabinol on the dopamine system. *Nature*. 2016;539(7629):369-377. PUBMED | CROSSREF

39. Atwood BK, Huffman J, Straiker A, Mackie K. JWH018, a common constituent of 'Spice' herbal blends, is a potent and efficacious cannabinoid CB receptor agonist. *Br J Pharmacol*. 2010;160(3):585-593. PUBMED | CROSSREF

40. Eggan SM, Lazarus MS, Stoyak SR, Volk DW, Glausier JR, Huang ZJ, et al. Cortical glutamic acid decarboxylase 67 deficiency results in lower cannabinoid 1 receptor messenger RNA expression: implications for schizophrenia. *Biol Psychiatry*. 2012;71(2):114-119. PUBMED | CROSSREF