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Involvement of nucleus accumbens SERCA2b in methamphetamine-induced conditioned place preference

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Abstract

Methamphetamine (METH) is a highly addictive psycho-stimulant that induces addictive behaviour by stimulating increased dopamine release in the nucleus accumbens (NAc). The sarco/endoplasmic reticulum calcium ion transport ATPases (SERCA or ATP2A) is a calcium ion (Ca2+) pump in the endoplasmic reticulum (ER) membrane. SERCA2b is a SERCA subtype mainly distributed in the central nervous system. This study used conditioned place preference (CPP), a translational drug reward model, to observe the effects of SERCA and SERCA2b on METH-CPP in mice. Result suggested that the activity of SERCA was significantly decreased in NAc after METH-CPP. Intraperitoneal SERCA agonist CDN1163 injection or bilateral CDN1163 microinjection in the NAc inhibited METH-CPP formation. SERCA2b overexpression by the Adeno-associated virus can reduce the DA release of NAc and inhibit METH-CPP formation. Although microinjection of SERCA inhibitor thapsigargin in the bilateral NAc did not significantly aggravate METH-CPP, interference with SERCA2b expression in NAc by adeno-associated virus increased DA release and promoted METH-CPP formation. METH reduced the SERCA ability to transport Ca2+ into the ER in SHSY5Y cells in vitro, which was reversed by CDN1163. This study revealed that METH dysregulates intracellular calcium balance by downregulating SERCA2b function, increasing DA release in NAc and inducing METH-CPP formation. Drugs that target SERCA2b may have the potential to treat METH addiction.

KEYWORDS
addiction, methamphetamine, sarco/endoplasmic reticulum calcium ion transport ATPases

1 | INTRODUCTION

Methamphetamine (METH) is one of the most commonly used and highly addictive illicit drugs in the world.[1] METH addiction is characterised by its uncontrollable overuse despite adverse consequences. Currently, there are no effective treatments for METH addiction.[2] Therefore, understanding the neurobiological mechanisms of METH addiction is of crucial importance. Most research on METH addiction in animals and humans focuses on the mesocorticolimbic dopamine system. The mesolimbic dopamine (DA) system is substantially associated with METH addiction.[3,4] Although METH has multiple effects on DA transmission, transient increased vesicular release and impaired

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DA uptake and vesicular packaging ultimately result in DA accumulation in nucleus accumbens (NAc),[5,6] the mechanism by which METH increases DA levels in NAc has not been fully elucidated. Previous research has found that intracellular calcium ion (Ca^{2+}) stores may be responsible for METH-induced DA increases. Blocking intracellular Ca^{2+} release from these stores inhibits METH effects on DA signals and related behaviour.[7]

The endoplasmic reticulum (ER) is the largest calcium reservoir in the cell and plays a vital role in maintaining intracellular Ca^{2+} homeostasis. Three ER membrane proteins mainly regulate the influx and outflow of Ca^{2+} from the ER, including inositol 1,4,5-trisphosphate receptors (IP3Rs), ryanodine receptors (RyRs) and sarco/endoplasmic reticulum calcium ion transport ATPases (SERCA or ATP2A). IP3Rs and RYRs transport Ca^{2+} out of the ER, whereas SERCA is a Ca^{2+} pump that transports Ca^{2+} from the cytoplasm into the ER. Furthermore, the upregulated function or expression of IP3Rs and RyRs has been observed in NAc of METH-addicted animals; inhibiting IP3Rs or RyRs suppresses the development of METH-rewarding effects.[8,9] These studies suggest that METH can increase intracellular Ca^{2+} concentration by activating IP3Rs and RyRs, which promote Ca^{2+} efflux from the ER. However, the role of SERCA in METH addiction remains unclear.

The SERCA pumps primarily regulate intracellular Ca^{2+} homeostasis, maintaining low cytosolic Ca^{2+} levels by pumping free Ca^{2+} into the ER lumen via ATP hydrolysis.[10,11] About 30% of free Ca^{2+} released into the cytoplasm by IP3R and RyRs is transported out, while 70% is pumped back into the ER by SERCA; therefore, it is an important Ca^{2+} pump.[12]

The SERCA are encoded by three distinct genes, SERCA1–3, resulting in 12 known protein isoforms with tissue-dependent expression patterns.[13] SERCA2b is one of the SERCA subtypes, which is mainly distributed in the central nervous system.[14] Although the structure and function of the SERCA have been comprehensively studied, their role in the central nervous system remains undetermined. Studies have shown that SERCA-mediated Ca^{2+} dyshomeostasis has been associated with neuropathological conditions, such as bipolar disorder, schizophrenia, Parkinson's disease and Alzheimer's disease.[15] Moreover, SERCA2 has also been associated with a hyperdopaminergic state in NAc.[16] However, whether SERCA increases DA release induced in NAc by METH use remains unclear. Therefore, we hypothesise that SERCA, especially the SERCA2b, may involve in METH-induced CPP.

This study investigated the roles of SERCA and SERCA2b in METH-induced conditioned place preference (CPP) model to identify and characterise the molecular mechanisms of SERCA that might be involved in METH addiction.

2 | MATERIALS AND METHODS

2.1 | Animals

Male Institute of Cancer Research (ICR) mice (weight = 25–35 g and age = 6 weeks) were purchased from Henan Sikebas Biotechnology Co., Ltd (Certificate No. Henan SCXK 2020–0005) and housed (n = 11–13 mice/cage) in a 12 h light/dark cycle, at 20–25°C, 45%–55% humidity and free access to food and water. ICR mice were widely used in biomedical research, and we used ICR mice in our previous studies,[17] so we used ICR mice in this study for the continuity of the study. The mice were allowed to adapt to the human touch for 1 week before treatment. To minimise the basal stress, all mice were gently handled twice daily before the behavioural experiment. All the animal experiments in this study followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Bengbu Medical College (Bengbu, Anhui, China).

2.2 | Drugs and antibodies

The SERCA agonists CDN1163 (HY-101455), inhibitor thapsigargin (HY-13433), polyethene glycol 300 (PEG 300, HY-Y0873) and tween-80 (HY-Y1891) were purchased from MedChemExpress, China. The dose of METH (1 mg/kg, i.p.) in the conditioning phase was established from a dose-dependent study conducted, where METH (1 mg/kg) produced a reliable and consistent CPP, and the doses of 1, 2.5, 5 and 10 mg/kg did not produce a dose-dependent change in place preference.[18] As higher doses did not result in greater place preference changes, we used a dose of 1 mg/kg in this study. This dose was consistent with previous studies, confirming that METH is a reward-related addictive drug.[19–22] METH was obtained from the Key Laboratory of Addiction Medicine, Bengbu Medical College. METH was dissolved in saline at a concentration of 1 mg/kg, and the injectable dose was 10 mL/kg (i.p.). Saline was purchased from Anhui BBCA Pharmaceutical Co., Ltd., and the injection dose of intraperitoneal saline in the control group was 10 mL/kg. CDN1163 or thapsigargin was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration (10 mg/mL) and then diluted with PBS to work concentrations (1 mM/5 mM of CDN1163 and 1 μM of thapsigargin, DMSO <20%). During the conditioning phase, 5 min before METH administration, CDN1163 (1 μL/side) or thapsigargin (1 μL/side) was bilaterally microinjected into the NAc. The control mice were administered with the same dose of PBS instead of the drugs. Alternatively, CDN1163 (10 and 50 mg/kg) or thapsigargin (1 mg/kg) was injected intraperitoneally 60 min before the administration of METH. The mouse monoclonal β-actin (A5441) antibody and ATP/GTP enzyme activity assay kits (MAK113-1KT) were acquired from Merck, Germany. DMSO was purchased from Beyotime, China. The human neuroblastoma SH-SY5Y cells (CL-0208) were provided by Priscilla, China. Dulbecco's modified eagle medium (DMEM) (BL304A) and foetal bovine serum (FBS) (FB25015) were purchased from Biosharp (China) and Clark (Germany), respectively. Mag-Fluo-AM fluorescence (GMS10267.1) was purchased from GENMED, USA. DAPI (4′,6′-diamidino-2-phenylindole) Staining Solution (BL105A) was acquired from Biosharp, China.

2.3 | CPP

The CPP apparatus (Zhenghua Software and Instruments, Anhui, China) comprised a square chamber of two equal-size compartments (A and B: 30 cm length × 25 cm width × 32 cm height) linked by a middle chamber (C: 11 cm length × 25 cm width × 32 cm height). Compartment A had black and white vertical stripes on the walls and equally spaced black vertical stripes at the bottom, whereas compartment B had all-black walls with evenly spaced black circular hole panels at the bottom. Compartment C was neutral, with white walls and equidistant black-striped floors. Sliding removable doors were used to separate these compartments. The animals' behaviour was recorded automatically via a video camera mounted on the ceiling above the centre of the apparatus, interfaced with a tracking system.

The METH-induced CPP paradigm was established, as described previously.[23] The place conditioning procedure has the following phases: habituation, preconditioning, conditioning and testing. During the habituation phase, mice were placed in the neutral compartment (C), and then the sliding doors were opened, allowing the animal to freely explore the apparatus for 30 min. In the preconditioning phase, mice were also placed in the neutral compartment (C), then the sliding doors were opened and mice could freely explore the apparatus for 15 min. The duration spent in each compartment was recorded. Mice with substantial unconditioned aversion (>720 s) for one of the compartments were excluded. Generally, two mice in each group would be excluded from the experiment for this reason. Specifically, the groups that excluded one mouse were as follows: the CPP group of Figure 4A, the Con group of Figure 4C, the Thapsigargin (1 μM) + CPP group of Figure 4D and the Con group of Figure 5D; the groups that excluded two mice were as follows: all groups of Figure 3A, the Con group and CPP group of Figure 3B, Con group and CDN1163 (1mM) + CPP group of Figure 4A, Con group of Figure 4B, CPP group and Thapsigargin (0.5 μM) + CPP group of Figure 4C, Con group and CPP group of Figure 4D, Con group and adeno-associated virus (AAV)-SERCA2b + CPP group of Figure 5C, AAV-vector+CPP group and AAV-SERCA2b shRNAi+CPP group of Figure 5D; the exclusion of three mice was CDN1163 (50 mg/kg) + CPP group in Figure 3B, CPP group and CDN1163 (5mM) + CPP of Figure 4B and AAV-vector+CPP group of Figure 5C. After preconditioning, mice were subjected to 8 days of conditioning. On the first day, the mice were i.p. injected with either METH (1 mg/kg) or saline (1 mL/kg) and then confined for 45 min in the nonpreferred compartment. The mice were placed on the nonpreferred compartment after being treated with METH for the purpose of maximising the probability of obtaining METH-induced CPP. On alternate days, mice were injected with saline and then immediately placed in the preferred compartment. This procedure was repeated four times during the conditioning phase. Twenty-four hours after the conditioning trial, the testing phase was initiated by placing the mice into neutral compartments (C), then opening the sliding doors and allowing the mice to explore the entire apparatus for 15 min, and the time spent by mice in each compartment was recorded. The CPP score was the time spent in the drug-paired compartment during the testing phase minus the time spent in that compartment during the preconditioning phase. METH- and saline-treated mice were individually housed in different cages under the same standard conditions (Figure 1).

2.4 | Intracerebral microinjection and agonist/inhibitor interventions

For cannula implantation and microinjection, mice were anaesthetised with 4% chloral hydrate under aseptic conditions via a stereotaxic instrument (RWD Life Science Co., Ltd) with the incisor bar set at 3.3 mm.

For NAc infusion, a guide cannula (26 gauge) was bilaterally implanted in the NAc (anteroposterior = +1.18 mm, mediolateral = ±1.0, dorsoventral = − 4.25 mm). One week postsurgical and anaesthesia recovery, mice were again placed in a stereotaxic apparatus for drug infusion. For bilateral microinfusions, 31-gauge injection needles (1 mm below the tip of the guide cannulae) connected to a 10 μL microsyringe mounted in the microinfusion pump (Harvard Apparatus, Inc.) were used. During the injection, the animals were gently restrained by hand.

SERCA agonist (CDN1163) or inhibitor (thapsigargin) was dissolved in DMSO to a stock concentration (10 mg/mL) and then diluted with PBS to work concentrations (1 mM/5 mM of CDN1163 and 1 μM of thapsigargin, DMSO <20%). During the conditioning phase, 5 min before METH administration (1 mg/kg, i.p.), CDN1163 (1 μL/side) or thapsigargin (1 μl/side) was bilaterally microinjected into the NAc. The control mice were administered with the same dose of PBS instead of the drugs. The infusion rate was 0.5 μL/min. Mice were given an additional 2 min to allow drug diffusion after injection.

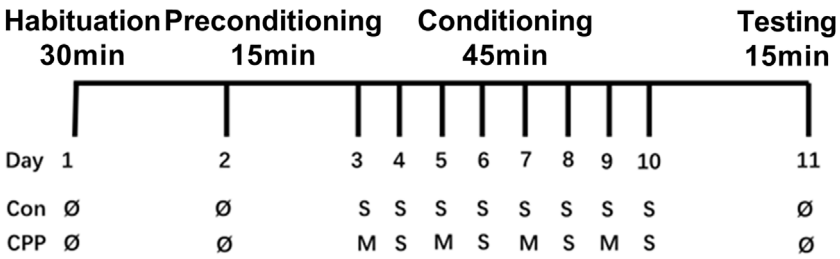

FIGURE 1 CPP training paradigm. ø, no injection. M, METH injection (1 mg/kg, i.p.). S, saline injection (1 mL/kg, i.p.). con, control, saline injection (10 mL/kg, i.p.); CPP, METH injection (1 mg/kg, i.p.). CPP, conditioned place preference; METH, methamphetamine

2.5 | Intervention of overexpression/interfering AAV

The SERCA2b overexpression/interfering AAV was purchased from Obio Technology Co., Ltd. Shanghai, China. For infecting mice NAc, 2.0×10^{13} IU/mL of AAV stocks were used. Briefly, mice were anaesthetised, and microinfusions were made, as mentioned in Section 2.4. AAV (1 μL/site) was microinjected into the bilateral NAc (anteroposterior = +1.18 mm, mediolateral = ±1.0, dorsoventral = − 4.25 mm) over 15 min, and the microinjection needle was retained for another 5 min for diffusion. Subsequent in vivo experiments were performed 2–3 weeks after the AAV injection.

2.6 | Subcellular fractionation

For Western blotting, SERCA activity and DA content analyses, mice were anaesthetised and sacrificed by decapitation immediately after METH-CPP on the seventh day of CPP conditioning, per the literature.[24] After euthanization, mice brains were immediately dissected and cut into coronal slices (1 mm thick) using a mice brain slicer (Braintree Scientific), and a blunt 17-gauge syringe needle was used to puncture the NAc. The homogenate was centrifuged at 1000 × g for 10 min at 4°C, and the supernatant was collected for subsequent analyses.

2.7 | Immunoblotting

Equal amounts of protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gels for electrophoresis and then transferred to the nitrocellulose membrane. Primary SERCA2 (1:1000 dilution) and β-actin (1:10000 dilution) antibodies diluted in TBS containing 5% nonfat dried milk and 0.05% tween-20 were used to determine the corresponding protein expression. Then secondary antibodies (HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG) were tagged with primary β-actin (1:20000 dilution) and SERCA2 (1:2000 dilution) antibodies. The immunoreactive bands were detected with a chemiluminescent substrate (RPN2232, GE Healthcare). The immunoreactive signals were quantified by quantity analysis software (Bio-Rad).

2.8 | Histology

Mice were deeply anaesthetised with chloral hydrate for transcardial perfusion, first with 0.9% saline and then with 4% paraformaldehyde in PBS. The brains were samples, fixed in 4% paraformaldehyde for 24 h, dehydrated for 3–5 days in a 30% sucrose/PBS solution, sliced coronally (30 mm thick) on a cryostat (Leica), stained with crystal violet and examined under light microscopy to detect the injection sites. Only animals with the right injection sites were included for data analysis.

2.9 | Measurement of SERCA activity

SERCA ATPase activity was assessed per the ATPase activity assay kits' instructions. SERCA ATPase hydrolyzes ATP into ADP and free phosphate. Free phosphate causes the malachite green reagent to form a stable dark green colorimetric product, representing the ATPase activity levels. Briefly, 50 μM of phosphate standard solutions (0, 10, 20, 25, 30 and 40 μL) were added to 96-well plates to generate 0 (blank). About 0, 12.5, 25, 31.25, 37.5 and 50 μM of standard phosphate were added (500, 1000, 1250, 1500 and 2000 pmol/well). The absorbance at 620 nm was detected to plot the phosphate standard curve. NAc tissue samples (20 mg) were homogenised with ice-cold assay buffer (200 μL), centrifuged at 14 000 r/min for 10 min at 4°C to remove insoluble materials, and then the supernatant was collected. The reaction mixture solution (30 μL/well) was incubated in a 96-well flat-bottom plate for 30 min at room temperature. To terminate the enzyme reaction, a reagent (200 μL) was added to each well for an additional 30 min at room temperature. Lastly, the absorbance of all the samples was determined at 620 nm.

2.10 | SH-SY5Y cell culture and measurement of calcium ion concentration in the ER

SH-SY5Y cells were cultured in DMEM augmented with 10% FBS and penicillin/streptomycin (100 units/mL) at 37°C with 5% CO_2 humidified incubator. After incubation, the media was refreshed with one without calcium and magnesium ions to observe the effect of CDN1163 on the ability of SERCA to transport Ca^{2+} into ER. Cells were first treated with METH (1 mM) for 10 min and then with CDN1163 (10 μM) for 10 min. The cells were then stained with Mag-Fluo-3AM fluorescence to determine ER Ca^{2+} concentration for 30 min at room temperature and darkness, mounted with DAPI for nuclear counterstaining, washed with PBS and then observed under an inverted microscope (Axio Observer Z1, Zeiss, Germany).

2.11 | Measurement of dopamine content

High-performance liquid chromatography (HPLC) was performed to measure DA content using a Waters Symmetryshield RP18 column (4.6 mm × 25 cm, 5 μm) (Waters, MA, USA) at a 25°C via the fluorescence detector of HPLC system (Shimadzu LC solution, Japan). In the chromatogram of DA hydrochloride standard, the peak time of DA at the absorption wavelength of 280 nm was detected. The peak areas of 0.5, 1, 2, 5, 10 and 20 μg/mL DA hydrochloride standard were detected, and the standard curve of DA concentration was plotted. Samples for DA content assessment were taken from the NAc of the mice, and the extraction method was as described in Section 2.6. above. Samples were eluted with a mobile phase chromatographic grade methanol at a flow rate of 0.8 mL/min, the injection volumes were 20 μl and the eluent was monitored at 280 nm.

3 | STATISTICAL ANALYSIS

This study used the SPSS 25.0 software and GraphPad Prism 8 for statistical analysis and graphing. For intergroup comparison, the independent sample *t*-test was performed. The measured data for multiple groups were analysed by one-way Analysis of Variance (ANOVA). Differences with $p < 0.05$ were considered statistically significant, and the results are presented as mean ± Standard Error of Mean (SEM).

4 | RESULTS

4.1 | Downregulated activity of SERCA in the NAc of METH-CPP mice

To determine if SERCA was involved in METH-induced CPP, its activity in the NAc was assessed after METH-CPP. A significantly reduced SERCA activity was observed in the NAc in the METH-CPP mice than in the control mice ($p < 0.05$) (Figure 2), suggesting that the activity of SERCA may be involved in the development of METH-CPP.

4.2 | Intraperitoneal injection of SERCA agonist CDN1163 prevented the formation of METH- CPP

The above results indicated that a reduction of the activity of SERCA accompanied METH-induced place preference induction. Currently, no specific agonists and antagonists of SERCA2b exist; therefore, to confirm the role of SERCA in METH-induced CPP, the effects of i.p. injection of SERCA agonist (CDN1163) and antagonist (thapsigargin) were assessed 10 min before each METH pairing. About 10 mg/kg of CDN1163 did not affect METH-CPP formation; however, 50 mg/kg significantly prevented CPP formation (CPP score: control group, 12.95 ± 22.63 s; METH group, 134.1 ± 22.47 s; METH

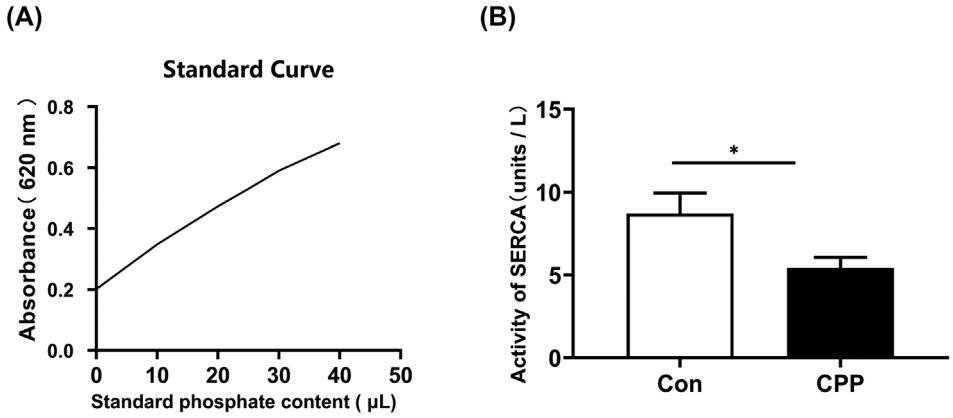

FIGURE 2 (A) The phosphate standard curve. (B) SERCA activity was significantly decreased in the NAc of METH-CPP mice. Error bars represent mean ± SEM, $n = 6–8$/group. *$p < 0.05$ compared with the control group, two-tailed Student's *t*-test. Con: control, saline (10 mL/kg, i.p.); CPP: METH injection (1 mg/kg, i.p.). CPP, conditioned place preference; METH, methamphetamine; NAc, nucleus accumbens; SERCA, sarco/endoplasmic reticulum calcium ion transport ATPases

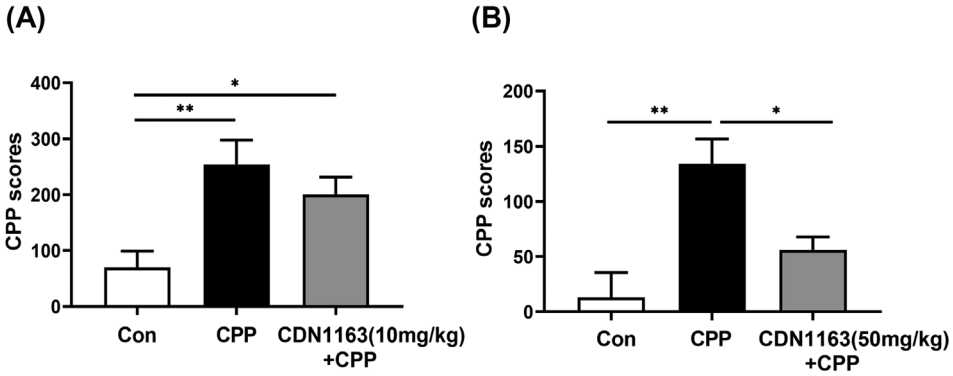

FIGURE 3 (A) 10 mg/kg intraperitoneal injection of CDN1163 did not significantly inhibit METH-CPP formation, whereas (B) 50 mg/kg injection significantly inhibits METH-CPP formation. Error bars represent mean ± SEM, $n = 11–13$/group. **$p < 0.01$, *$p < 0.05$, one-way ANOVA with Newman–Keuls post hoc test. Con: control, saline injection (10 mL/kg, i.p.); CPP: METH injection (1 mg/kg, i.p.). CPP, conditioned place preference; METH, methamphetamine; NAc, nucleus accumbens

+CDN1163 [50 mg/kg] group, 56.1 ± 11.81 s; $F(2,34) = 9.554$, $p < 0.001$] (Figure 3). Furthermore, this study found that i.p. injection of 1 mg/kg dose of thapsigargin caused death in mice. Because SERCA plays an important role in maintaining the function of skeletal muscles, it was presumed that death caused by thapsigargin might be linked with the disruption of myocardial and skeletal muscle functions. Therefore, data on SERCA2b antagonist thapsigargin are not presented.

4.3 | Intra-NAc injection of SERCA agonist CDN1163 prevented the formation of METH- CPP

To further elucidate how SERCA is associated with METH-induced CPP, the brain stereotaxic technique was used to inject CDN1163 directionally into the bilateral NAc of mice 5 min before each METH pairing. The 1 mM/side of CDN1163 did not affect METH CPP formation; however, 5 mM/side significantly prevented CPP formation (CPP score: control group, 79.35 ± 20.78 s; METH group, 228.1 ± 39.48 s; METH+CDN1163 (50 mg/kg) group, 130.6 ± 25.28 s; $F(2,35) = 6.642$, $p < 0.05$). This indicated that CDN1163 might dose-dependently interfere with METH-induced CPP formation (Figure 4A,B).

It has been indicated previously[25–27] that 0.5–1 μM thapsigargin is a commonly used concentration for inhibiting SERCA in intact cells; based on this, two groups of treatments were created: (1) microinjected with 0.5 μM and (2) with 1 μM thapsigargin into the Nac. The results showed that thapsigargin did not increase the mouse's addictive behaviour compared to the CPP group (Figure 4C,D), possibly because the SERCA family are important Ca^{2+} pumps that maintain cell function. When thapsigargin completely and rapidly inhibits SERCA activity, it inhibits neural function in NAc.[28] Therefore, the interfering SERCA2b AAV was used to reduced SERCA2b expression, and it could promote METH-CPP (Figure 5).

4.4 | Overexpressing SERCA2b significantly inhibited the formation of METH-CPP, and knockdown of SERCA2b exacerbated METH-CPP

To validate SERCA2b activity, a brain stereotaxic technique was used to inject AAV into the NAc of mice to overexpress or knockdown SERCA2b. Overexpressing SERCA2b significantly inhibited METH-CPP formation (CPP score: control group, 61.03 ± 24.45 s; AAV-vector+CPP group, 251.1 + 33.76 s; AAV-SERCA2b + CPP group, 134.7 ± 44.41 s; $F(2,32) = 7.894$, $p < 0.01$), whereas its knockdown had the opposite effects (CPP score: control group, −4.846 ± 18.08 s; AAV-vector+CPP group, 96.09 + 20.84 s; AAV-SERCA2b + CPP group, 159.0 ± 21.92 s; $F(2, 35) = 16.51$, $p < 0.001$) (Figure 5).

4.5 | SERCA2b overexpression significantly decreased METH-induced increased DA levels, whereas SERCA2b knockdown increased DA levels in NAc

The DA levels in the NAc were measured after CPP on the 7th day of CPP conditioning. As shown in Figure 6, the NAc DA levels were significantly higher in the CPP group mice than the control mice, and

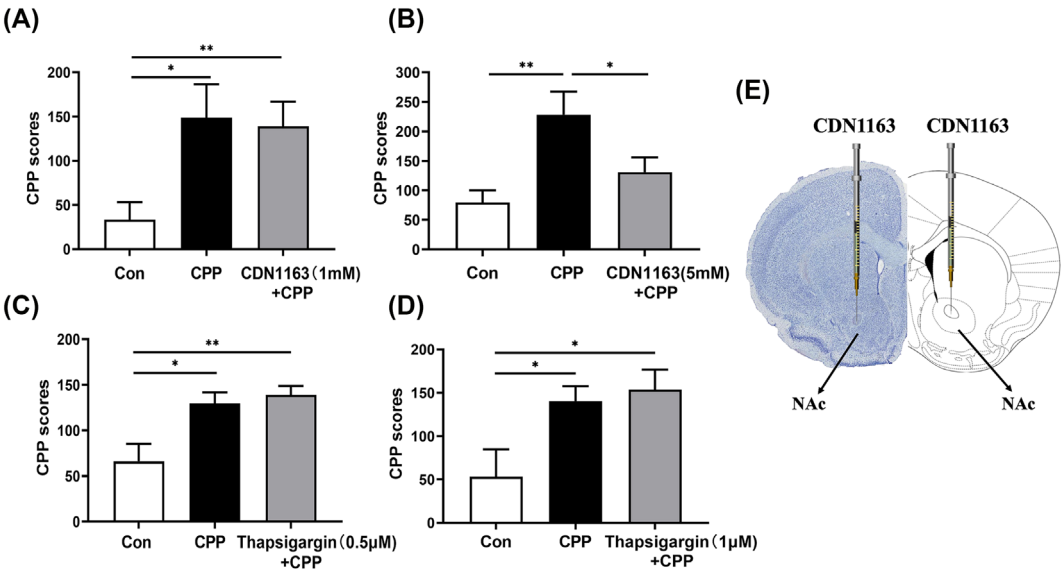

FIGURE 4 (A) The injection of CDN1163 (1 mM/side) did not affect METH CPP formation. (B) However, the injection of 5 mM/side significantly prevented CPP formation. (C, D) The injection of thapsigargin (0.5 or 1 μM/side) did not affect METH-CPP formation. (E) Nissl staining and injection of sites in the NAc. Error bars represent mean ± SEM, $n = 11–13$/group. $^{**}p < 0.01$, $^{*}p < 0.05$, one-way ANOVA with Newman–Keuls post hoc test. Con: control, saline injection (10 mL/kg, i.p.); CPP: METH injection (1 mg/kg, i.p.). CPP, conditioned place preference; METH, methamphetamine; NAc, nucleus accumbens

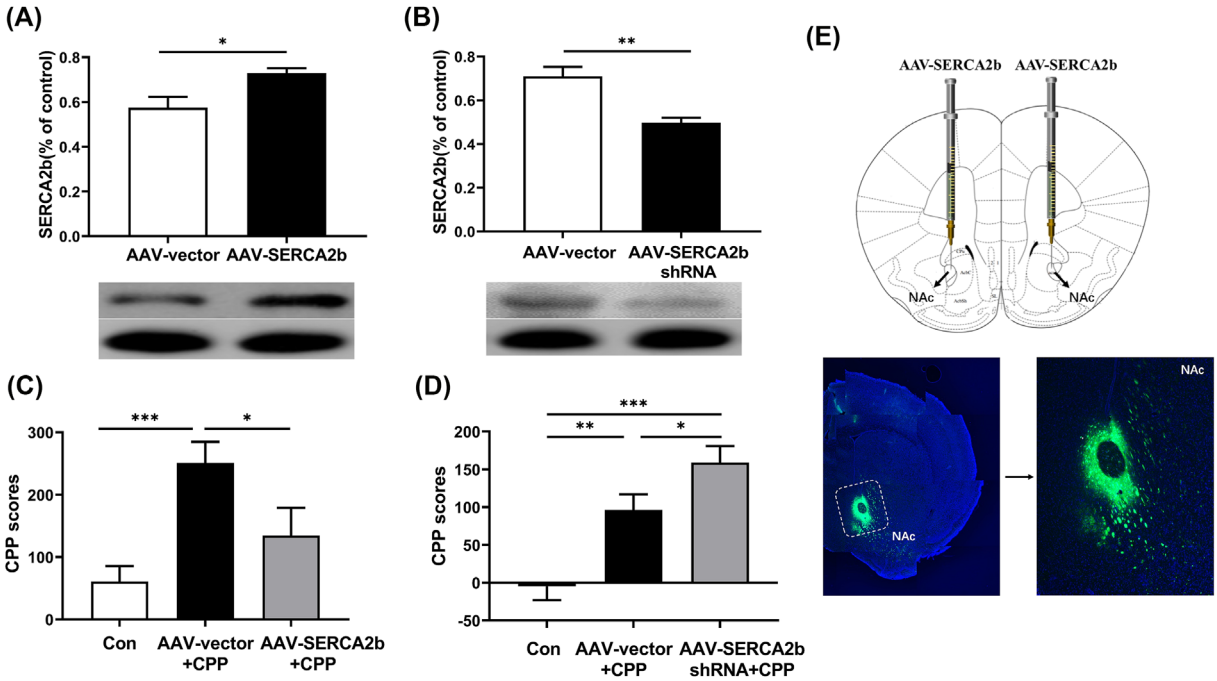

FIGURE 5 (A) AAV-induced SERCA2b overexpression increased SERCA2b expression; (B) SERCA2b interference AAV knockdown SERCA2b expression. (C) Overexpressing SERCA2b significantly inhibited the formation of METH CPP. (D) SERCA2b knockdown exacerbated METH-CPP behaviour. (E) representative image of in vivo AAV-infected NAc visualised by fluorescence microscope. Error bars represent mean ± SEM, $n = 11–13$/group. $^{**}p < 0.01$, $^{*}p < 0.05$, two-tailed Student's *t*-test or one-way ANOVA with Newman–Keuls post hoc test. Con: control, saline injection (10 mL/kg, i.p.). AAV, adeno-associated virus; CPP, conditioned place preference; METH, methamphetamine; NAc, nucleus accumbens; SERCA, sarco/endoplasmic reticulum calcium ion transport ATPases

SERCA2b overexpression markedly decreased CPP-induced DA levels than the control group; however, SERCA2b knockdown increased DA levels in Nac. The increase of DA release in the NAc is an important factor promoting METH addiction. These results indicated that SERCA2b overexpression might inhibit METH-CPP formation through decreased NAc DA levels induced by METH (Figure 6).

4.6 | SERCA2b agonist CDN1163 increased METH-induced decreased calcium concentration in the ER of SH-SY5Y cells

METH (1 mM, 10 min) decreased ER Ca^{2+} concentration in SH-SY5Y cells; however, this METH-induced decreased Ca^{2+} concentration was reversed by CDN1163 (10 μM, 10 min) (Figure 7).

5 | DISCUSSION

METH is a strongly addictive natural psychostimulant, and currently, no effective medicines exist to treat METH addiction. In this study, the function of SERCA was explored using the CPP model, a classic animal model used for examining the reward-associated effects of addictive drugs. It was revealed that METH-CPP significantly reduced SERCA function in NAc, and improving the SERCA function or SERCA2b expression in NAc could inhibit METH-CPP formation. This work provides a reference for exploring the mechanism of METH addiction.

SERCA is a Ca^{2+} pump that transports Ca^{2+} from the cytoplasm into the ER to maintain low cytosolic Ca^{2+} levels.[29] Previously, most SERCA family research was focused on the cardiovascular field[30]; however, in recent years, they have been associated with neuropathological conditions, such as bipolar disorder, schizophrenia, Parkinson's and Alzheimer's disease.[15,31] SERCA belongs to the family of P-type ATPases, which includes a variety of membrane pumps that utilise ATP hydrolysis and a phosphorylated enzyme intermediate to transport ions across cellular membranes.[32,33] In eukaryotes, three distinct genes, SERCA1–3 (or ATP2A1–3 in humans), encode 12 known SERCA protein isoforms, primarily via alternate splicing.[13] SERCA2b has also been found to be the only isoform expressed in neuronal microsomes, synaptic plasma membrane vesicles and synaptosomes.[14] Given its expression, SERCA2b is considered an ER housekeeping enzyme crucial for maintaining intracellular Ca^{2+} homeostasis in the CNS.[15]

No reports are indicating the direct involvement of SERCA in addiction; some studies suggest that the two might be related. (1) Brain-specific loss-of-function of SERCA2 causes a hyperdopaminergic state in NAc, which might be explained by the conditional SERCA2 knockout in the brain, causing prolonged cytosolic Ca^{2+} transients, possibly resulting in enhanced DA signalling.[16]

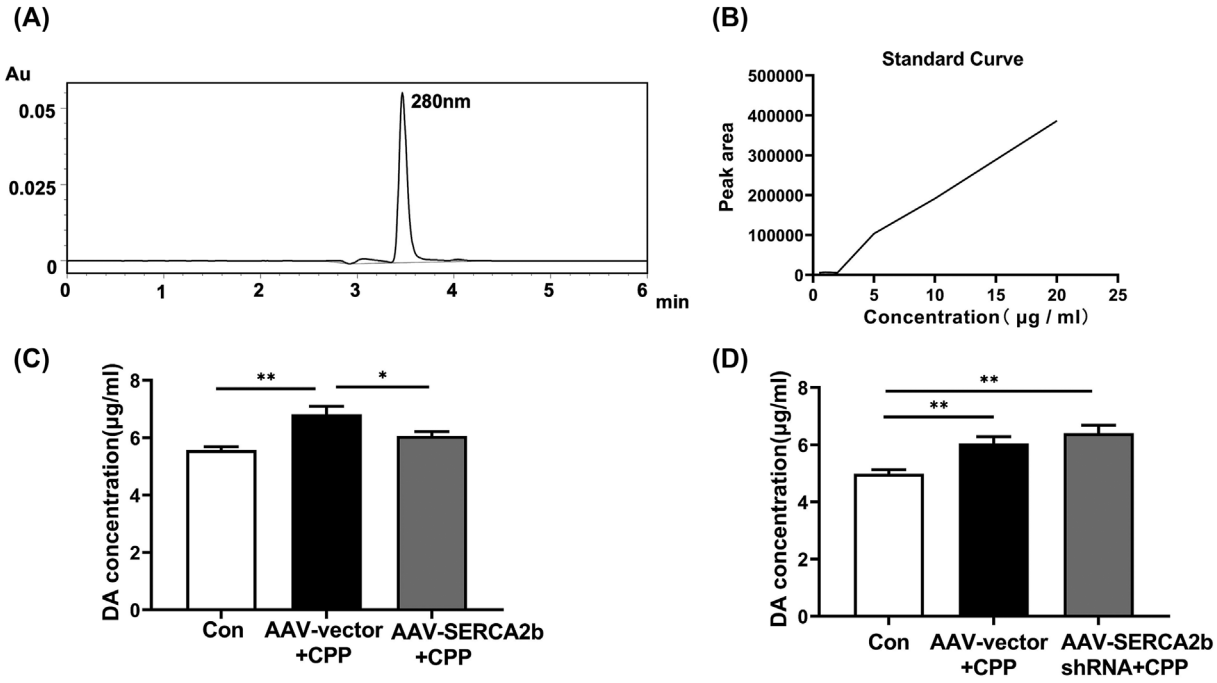

FIGURE 6 (A) The dopamine (DA) level in NAc was detected by HPLC. (B) DA concentration standard curves. (C) SERCA2b overexpression (AAV-SERCA2b) significantly decreased METH-induced DA levels. (D) SERCA2b knockdown (AAV-SERCA2b shRNA) increased DA levels in NAc than the control group. Error bars represent mean ± SEM, $n = 11–13$/group. $^{**}p < 0.01$, $^{*}p < 0.05$, one-way ANOVA with Newman–Keuls post hoc test. Con: control, saline injection (10 mL/kg, i.p.). AAV, adeno-associated virus; CPP, conditioned place preference; DA, dopamine; METH, methamphetamine; NAc, nucleus accumbens; SERCA, sarco/endoplasmic reticulum calcium ion transport ATPases

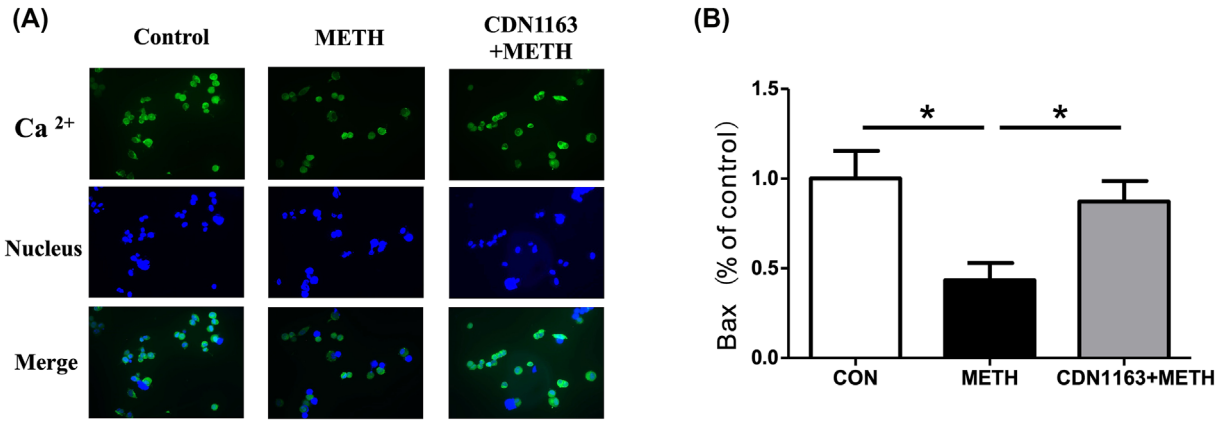

FIGURE 7 (A) Endoplasmic reticulum (ER) calcium ion concentration (Mag-Fluo-3AM, green) counterstained with the nucleus (DAPI, blue) in the SH-SY5Y cells. (B) CDN1163 reversed METH-induced decreased ER calcium concentration in SH-SY5Y cells. Error bars represent mean ± SEM, $n = 6–8$/group. $^{**}p < 0.01$, $^{*}p < 0.05$, one-way ANOVA with Newman–Keuls post hoc test. Con: control, saline injection (10 mL/kg, i.p.). METH, methamphetamine

Increased DA release in the NAc is a common feature of multiple substance addictions. (2) Sigma-1 receptor, a ligand-operated ER membrane protein, can be activated by METH, which promotes Ca^{2+} to flow out of ER and participates in the formation of METH addiction.[34] Sigma-1 receptor activation also promotes SERCA2 degradation through ER-associated degradation.[35] Moreover, its antagonists AC927 and CM156 can relieve METH addiction.[36,37] The above studies indirectly indicate that the Sigma-1 receptor may participate in the formation of METH addiction by downregulating SERCA2 expression. (3) Furthermore, it has been found that chronic CDN1163 administration induced anxiogenic and depressive-like behaviour in mice,[38] which are associated with decreased DA release in the NAc,[39–41] it can be inferred that the use of CDN1163 might reduce DA release in the NAc.

This study found that SERCA activity was downregulated in NAc of mice addicted to METH. The Ca^{2+} concentration in ER was

decreased in METH-treated SH-SY5Y cells, which could be reversed by CDN1163. Because no agonists and antagonists target only SERCA2b, the effects of CDN1163 and thapsigargin were assessed. The ability of CDN1163 to effectively activate SERCA has been demonstrated in previous studies; for instance, Kang et al. reported that CDN1163 increased SERCA levels in microsomes, SERCA-mediated Ca^{2+} uptake in vitro (10 μM) and enhanced SERCA activity in vivo (50 mg/kg, i.p. injection).[42] The 1 μM of thapsigargin has a potent antagonistic effect on the SERCA and is widely used to study Ca^{2+} signalling in vitro.[43]

These above results suggest that METH may reduce the influx of intracellular Ca^{2+} into ER by inhibiting SERCA activity, thereby increasing the intracellular Ca^{2+} concentration. Furthermore, it has been reported that decreased ER Ca^{2+} concentration can activate store-mediated Ca^{2+} flow into the cell, further increasing its intracellular concentration.[44]

The increase of intracellular Ca^{2+} could affect METH addiction by increasing the release of DA in the NAc. For example, research has found that amphetamine could promote the release of DA in the NAc via increased intracellular Ca^{2+} release.[45] Additionally, it has been suggested that increased intracellular Ca^{2+} increases DA release through unknown mechanisms.[46,47] The increase in DA release in NAc is an important factor promoting METH addiction.[48] This study found that METH increases DA in the NAc of addicted mice, whereas SERCA2b overexpression by AAV could reverse this METH-induced DA increase. Intraperitoneal and intra-NAc injections of CDN1163 (SERCA agonist) and SERCA2b overexpression in NAc significantly prevented the formation of METH-CPP. Furthermore, the intra-NAc injection of thapsigargin (SERCA antagonist) did not affect METH-CPP formation, and AAV-mediated SERCA2b knockdown in NAc exacerbated METH CPP.

These results suggest that METH may induce CPP formation by downregulating SERCA2b function, reduced ER and increased intracellular Ca^{2+} concentrations, and increased DA release in the NAc. Moreover, improving the SERCA function or SERCA2b expression can reverse METH-CPP formation through this pathway. In summary, this study suggests that SERCA2b affects METH-CPP formation by regulating intracellular Ca^{2+} levels; therefore, SERCA2b may be an efficient target for developing drugs against METH addiction.

5.1 | Limitation

The intracellular Ca^{2+} level is also influenced by the influx of extracellular Ca^{2+},[49] and this study only discusses the role of intracellular Ca^{2+} in METH-CPP formation, excluding the involvement of extracellular Ca^{2+} influx. Furthermore, Ca^{2+} has various effects on cells; for example, the Ca2+/calmodulin-dependent protein kinases (CaMKs, or calmodulin kinases) play key roles in neuronal transmission, synaptic plasticity, circuit development, addiction and cognition,[49,50] which were not considered. In addition, the mice used in this study were all male, which may have resulted in some gender bias in the experimental results. Therefore, further research is required.

AUTHOR CONTRIBUTIONS

The original draft of the work was written by Yujing Wang, Fan Duan and Junda Li. DongLiang Jiao and XiaoChu Zhang revised the text critically for essential intellectual content. All authors participated in writing the manuscript, approved the final version, and made significant contributions to its preparation.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no competing interests.

DATA AVAILABILITY STATEMENT

Research data are not shared.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Bengbu Medical College (Bengbu, Anhui, China).

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