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Protein kinase G regulates β-synuclein in response to repeated exposure to cocaine in the rat dorsal striatum in a Ca^{2+}-dependent manner

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HIGHLIGHTS

- Repeated cocaine upregulated β-synuclein expression in the rat dorsal striatum.
- Upregulation of β-synuclein was decreased by the depletion of Ca^{2+}.
- Blockade of NMDA receptors decreased elevated β-synuclein by repeated cocaine.
- Blockade of ryanodine receptors decreased β-synuclein elevated by repeated cocaine.

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ABSTRACT

Protein kinase G (PKG) activation plays a crucial role in neuronal plasticity after repeated exposure to cocaine in the dorsal striatum. The present study investigated the characteristics of β-synuclein expression by PKG activation after repeated cocaine administration in the rat dorsal striatum. The results demonstrated that repeated, but not acute, exposure to cocaine (20 mg/kg) once a day for 7 consecutive days significantly upregulated expression of β-synuclein. Furthermore, this upregulation was decreased by the depletion of Ca^{2+}, but not blockade of Na^{+} influx. Blockade of *N*-methyl-D-aspartate (NMDA) receptors and ryanodine-sensitive Ca^{2+} channels also decreased the elevation of β-synuclein expression by repeated cocaine administration. Inhibition of neuronal nitric oxide synthase, which can activate PKG, did not alter the expression of β-synuclein elevated by repeated cocaine administration. These findings suggest that the expression of β-synuclein can be regulated by Ca^{2+}-dependent PKG activation via stimulation of NMDA receptors and voltage-operated Ca^{2+} channels in the endoplasmic reticulum in the dorsal striatum.

1. Introduction

Synuclein is a soluble protein associated with a variety of synaptic plasticity in neurons [2,22]. Three subtypes of synuclein can be identified by their amino acid sequences. Specifically, α- and γ-synucleins commonly contain seven repeated consensus amino acid motifs (KTKEGV) in their N-terminus, while β-synuclein contains six repeated motifs in the N-terminus, but lacks 11 amino acids in the central region, which is the non-amyloid beta component of α-synuclein [8]. The α- and β-synucleins are mainly expressed in the neocortex, striatum, thalamus and hippocampus, while γ-synuclein is found in the red nucleus, substantia nigra reticulate, anterior commissure and peripheral nervous system [3,5,9,22]. Among these three types of synucleins, β-synuclein is known to play a crucial role in antagonizing neurotoxicity caused by α-synuclein in the central nervous system. For example, β-synuclein prevents neurotoxicity by blocking the aggregation of α-synucleins in a transgenic mouse model [10].

Pharmacologically, cocaine is an indirect dopamine agonist that elevates extracellular dopamine concentrations by blocking dopamine reuptake to its terminals in the dorsal striatum. Elevation of dopamine concentrations subsequently upregulates glutamate release into the dorsal striatum via trans-synaptic activation through basal ganglia circuitry [23]. Previous study has shown that repeated exposure to cocaine increases the efflux of nitric oxide (NO) in the dorsal striatum [14]. Elevation of NO efflux then upregulates cGMP formation followed by protein kinase G (PKG) activation in the dorsal striatum in a Ca^{2+}-dependent manner [15,24]. Taken together, these findings suggest that repeated cocaine administration activates PKG through glutamate

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receptor-mediated Ca^{2+} signaling cascades in which synuclein plays a crucial role in synaptic activity as a downstream regulator of PKG. Therefore, in this study, we determined the characteristics of β-synuclein expression, which can be regulated by Ca^{2+}-dependent PKG activity after repeated exposure to cocaine in the dorsal striatum, a key structure associated with drug addiction in the basal forebrain.

2. Materials and methods

Adult male Sprague–Dawley rats (230–250 g each) were purchased from Hyo-Chang Science (Daegu, Korea). Animals were housed in pairs in a controlled environment during the experimental treatments. Food and water were provided ad libitum and the rats were maintained on a 12 h light/dark cycle. Animals were adapted to the environment for 5 days before starting the experiments. On the day of the experiment, injections were administered in the home cage in a quiet room to minimize stress to the animals. All animal procedures were approved by the Institutional Animal Care and Use Committee and performed in accordance with the provisions of the NIH Guide for the Care and Use of Laboratory Animals.

Cocaine (Balgopia, Louvain-La-Neuve, Belgium) was dissolved in physiological saline (0.9% sodium chloride, NaCl) and injected in a 1 ml/kg volume of solution. Each rat received cocaine intraperitoneally (i.p.) once a day for 7 consecutive days. With the exception of xestospongin C (Millipore Bioscience Research, Billerica, MA, USA), all pharmacological drugs were purchased from Tocris Bioscience (Bristol, UK). The concentrations of drugs used were determined from previous studies [1,6,12,15,21]. The PKG inhibitor, KT5823 (2 nmol), the neuronal nitric oxide synthase (nNOS) inhibitor, N^{ω}-propyl (0.1 nmol), the Ca^{2+} chelator, BAPTA-AM (50 pmol), the Na^{+} channel blocker, tetrodotoxin citrate (TTX, 1 pmol), the *N*-methyl-D-aspartate (NMDA) receptor antagonists, AP5 (2 nmol) and MK801 (2 nmol), the ryanodine-sensitive Ca^{2+} channel blocker, dantrolene (20 nmol), the L-type voltage operated Ca^{2+} channel blocker, nifedipine (60 nmol), and the IP_3-sensitive Ca^{2+} channel blocker, xestospongin C (0.004 nmol), were dissolved in artificial cerebro-spinal fluid (aCSF) containing (mM) 123 NaCl, 0.86 $CaCl_2$, 3.0 KCl, 0.89 $MgCl_2$, 0.50 NaH_2PO_4, and 0.25 Na_2HPO_4 aerated with 95% O_2/5% CO_2 (pH 7.2–7.4) or dimethylsulfoxide (DMSO). All drugs were freshly dissolved in aCSF/DMSO before the experiments and injected into the right dorsal striatum. aCSF/DMSO mixture solution was injected into the same area of the brain in each control group.

The rats were anesthetized with 8% chloral hydrate (5.8 ml/kg, i.p.), then placed in a stereotaxic apparatus. Under aseptic conditions, a 23-gauge stainless steel guide cannula (inner diameter: 0.29 mm, 10 mm in length) was implanted 1 mm anterior to the bregma, 2.5 mm right of midline and 4 mm below the surface of the skull. The guide cannula was sealed with a stainless steel wire of the same length. Rats were allowed at least 5 days to recover from surgery. On the day of the experiment, the inner steel wire was replaced with a 30-gauge stainless steel injection cannula (inner diameter: 0.15 mm) with a length of 12.5 mm that protruded 2.5 mm beyond the guide cannula. Drug solutions were infused through the injection cannula to the central part of the right dorsal striatum 5 min before saline or cocaine injection in a total volume of 1 μl at a rate of 0.1 μl/min in freely moving rats. The progress of injection was monitored by observing the movement of a small air bubble through a length of precalibrated PE-10 tubing inserted between the injection cannula and a 2.5 μl Hamilton microsyringe (Fisher Scientific, Pittsburgh, PA, USA). After completion of the injection, the injector was left in place for an additional 5 min to prevent any possible backflow of the solution along with the injection tract. The physical accuracy of the injection was verified by the reconstruction of microinjection placements. The possibility of gliosis caused by the implantation of guide cannula and the infusion of drugs dissolved in DMSO/aCSF was verified using Nissl staining (data not shown).

Two-dimensional electrophoresis (2-DE) was conducted as previously described [13]. The brain samples of each group ($n = 5$) were pooled together and homogenized. For visualization, separated gels were incubated overnight in fixing solution containing 45% methanol and 5% phosphoric acid. The fixed gels were then stained with staining solution including 0.1% (w/v) Coomassie brilliant blue G250, 17% (w/v) ammonium sulfate, 3.6% phosphoric acid and 34% methanol for a day. Gels containing excess dye were destained with washing solution including 1% acetic acid and 15% methanol. The stained gels were then scanned using an ImageMaster Scanner, after which they were analyzed three times with ImageMaster 2D Platinum 6.0. The abundance of protein spots was determined by the area of the spot multiplied by the density and referred to as the volume. The spot volume was normalized to the total protein detected in each gel using the same software mentioned above. A 30% decrease or 100% increase in protein abundance was taken as indicating a difference between groups. Differently expressed proteins in each group were identified using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). The selected protein spots from the gels were destained with 50% acetonitrile in 25 mM ammonium bicarbonate (pH 8.0) three times for 15 min each, after which the spots were dehydrated with 100% acetonitrile (ACN). For in-gel digestion, the spots were rehydrated in 15 μl trypsin solution and incubated at 37 °C overnight. The supernatant was subsequently collected, after which the peptides were extracted with 5% trifluoroacetic acid in 50% ACN and dried. The extracted peptides were then desalted using ZipTips C18 resin and eluted with matrix solution (α-cyano-4-hydroxycinnamic acid in 50% ACN and 0.1% trifluoroacetic acid), after which peptide mass spectra were acquired on a Voyager-DE STR MALDI-TOF-MS. The peptide masses were analyzed by the Mascot program (http://www.matrixscience.com) using NCBInr databases. The identified proteins were characterized using the web sites http://ebi.uniprot.org or http://www.expasy.org.

Western blot analysis was performed as previously described [7]. Briefly, protein concentrations of the supernatant were determined using a BioRad Protein Assay (BioRad Laboratories, Hercules, CA, USA) based on the Bradford method and the supernatants were resolved using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), after which separated proteins were transferred to a nitrocellulose membrane. The membrane was then blocked with blocking buffer containing 5% skim milk and separately probed with primary antiserum against β-synuclein (Abcam, Cambridge, MA, USA) or β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:2000 dilution overnight at 4 °C on a shaker. Next, the membrane was incubated with the appropriate secondary antiserum (KPL, Gaithersburg, MD, USA) at a dilution of 1:1000 for 1 h at room temperature. Membranes containing immunoreactive protein bands were developed using Wastsave (Abfrontier, Seoul, Korea) on X-ray films. Differences in the number of immunoreactive pixels per measured area between groups after Western immunoblotting was determined by one-way ANOVA followed by Tukey's honestly significant difference test using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). All data are expressed as the mean ± SEM for each group ($n = 4$–5 per group). The level of statistical significance was set at $p < 0.05$.

3. Results

The first experiment was conducted to identify proteins regulated by PKG activation after repeated systemic cocaine injections