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Research report

Protracted cocaine withdrawal produces circadian rhythmic alterations of phosphorylated GSK-3β in reward-related brain areas in rats

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ABSTRACT

Protracted cocaine withdrawal can extend for months and contribute to cocaine seeking and relapse. However, no previous studies have reported the manifestation of protracted withdrawal from chronic cocaine in rats. Glycogen synthase kinase 3β (GSK-3β) can phosphorylate PER2, CRY2, Rev-erbα, and BMAL1 in mammals. The circadian rhythmic expression of GSK-3β in reward-related brain areas is unclear. We examined rodent behaviors and circadian disturbances of GSK-3β expression during 30 days of protracted cocaine withdrawal. The behavioral tests included open field, elevated plus maze, weight gain, and sucrose preference tests performed 3, 10, and 30 days after stopping cocaine. At these three assessment points, we collected brain samples every 4 h for 24 h to examine the circadian rhythmic expression of GSK-3β. Decreased locomotor activity, weight loss, decreased sucrose consumption on day 3, and increased time spent in the open arms of the elevated plus maze on day 10 after cocaine administration were found. Blunted circadian rhythms of phosphorylated GFK-3β (pGSK-3β) persisted for at least 30 days in all examined brain areas, with the exception of 10 days in the suprachiasmatic nucleus (SCN) and nucleus accumbens (NAc). The expression of pGSK-3β decreased in the SCN and increased in the NAc and ventral tegmental area persisted for at least 30 days, whereas in the prefrontal cortex decreased during withdrawal for 10 days but then reversed to abnormally high levels with protracted withdrawal. These long-lasting changes disrupted circadian rhythms and produced abnormal levels of phosphorylated GSK-3β protein in reward-related brain circuits, which may play a role in protracted cocaine withdrawal and contribute to relapse.

1. Introduction

Cocaine dependence continues to be a significant public health concern. Data from the 2009 European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) Annual Report indicated that the number of Europeans who had used cocaine in the past month has remained relatively stable [1]. Cessation of regular cocaine use is thought to result in withdrawal symptoms, such as dysphoric mood, changes in appetite, fatigue, vivid or unpleasant dreams, and increased or decreased psychomotor activity and sleep (APA, 2000). Withdrawal symptoms occurring within a few hours to several days following cessation of repeated cocaine use is believed to contribute to relapse to cocaine use [2,3].

One early study showed that individuals experience three distinct temporal phases ("crash," "withdrawal," and "extinction") after cessation of cocaine use [4]. The crash occurs within hours to a few days after cocaine use and is characterized by profound fatigue, lethargy, and depression. Withdrawal is characterized by milder symptoms and craving for cocaine 1–10 weeks after cessation of cocaine use. Extinction lasts indefinitely and is characterized primarily by a recurrent desire or craving for cocaine. Many subsequent studies on cocaine abusers or patients undergoing cocaine detoxification found that withdrawal symptoms were highest during the first few days of abstinence, declined linearly thereafter [5–9], and included negative mood, higher resting heart rate [10,11], sleep disturbances, and altered dopaminergic function [7,12], although these results have not been consistently demonstrated [5,6]. Cocaine withdrawal has recently been defined as comprising two stages [13]. The first stage, acute withdrawal, was

Abbreviations: GSK-3β, glycogen synthase kinase 3β; SCN, suprachiasmatic nucleus; PFC, prefrontal cortex; NAc, nucleus accumbens; VTA, ventral tegmental area.

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defined as the period shortly following the end of cocaine administration (sometimes referred to as the "crash") when cocaine blood levels begin to decrease (i.e., 5–24 h after the last dose of cocaine). The second stage, prolonged withdrawal, was defined as the 24 h time period after the last dose of cocaine when no appreciable cocaine levels are detectable in the blood. We think the latter stage is reasonable. However, the manifestation and neurobiological alterations of protracted withdrawal from chronic cocaine administration in rats are not clear.

Glycogen synthase kinase 3 (GSK-3), a serine/threonine kinase, has been shown to play various roles in protein synthesis, cell proliferation, cell differentiation, microtubule dynamics, cell motility, and neuronal apoptosis [14–16]. It is a ubiquitous kinase in brain and is associated with a variety of neurological disorders, such as Alzheimer's disease, bipolar disorder, Huntington's disease, and other neurodegenerative disorders [17,18]. It has been shown to also be involved in circadian rhythm regulation. In *Drosophila*, a reduction in GSK-3 activity by genetic manipulation causes period lengthening [19], in contrast to findings in mammalian cell cultures [20]. GSK3β, an isoform of GSK3, is known to phosphorylate PER2, CRY2, Rev-erbα, and BMAL1 in mammals [21–24]. GSK-3β-mediated phosphorylation leads to proteasomal degradation of CRY2 [22] and BMAL1 [24] and stabilization of Rev-erbα [23]. GSK-3β activity has been demonstrated in the nucleus accumbens (NAc) core but not NAc shell and mediates the initiation and expression of cocaine-induced locomotor sensitization [25] associated with the long-term neuroadaptations produced by repeated cocaine [26]. Phosphorylation of GSK3 was significantly reduced after 14 days of cocaine administration in the amygdala [27]. Previous studies showed that subchronic cocaine administration altered the circadian pattern of feeding behavior in rats [28]. Cocaine sensitization and reward are under the influence of circadian genes and rhythm [29]. However, alterations in circadian rhythmic phosphorylation of GSK3β in reward-related brain areas during protracted withdrawal from cocaine in rats are unknown.

The present study explored the manifestations of protracted withdrawal from chronic cocaine administration and the expression of GSK3β kinase in the suprachiasmatic nucleus (SCN) and reward-related brain areas in cocaine-treated rats. Because of the roles of GSK3β in circadian rhythmic regulation, we hypothesized that the manifestations of protracted cocaine withdrawal may not persist for a long time, whereas the marked changes in the circadian expression of GSK3β kinase in various brain areas related to addiction and reward may be sustained.

2. Materials and methods

2.1. Animals and drugs

One hundred sixty-eight male Sprague-Dawley rats were purchased from the Center of Laboratory Animal Science, Peking University Health Science Center. The rats weighed 270–310 g at the start of the study. Rats were group housed and maintained on a 12 h/12 h light/dark cycle (08:00 lights on; 20:00 lights off). Food and water were available *ad libitum* in the home cages. Rats were acclimated to handling and allowed to adapt for a minimum of 7 days before the start of the experiment. Rats received chronic administration of cocaine to induce cocaine withdrawal signs. Cocaine hydrochloride was obtained from Qinghai Pharmaceuticals (Xining, China), dissolved in saline, and intraperitoneally (i.p.) injected in a volume of 1 ml/kg.

2.2. Animal treatments and behavioral measurement

The rats were injected with cocaine (20 mg/kg, i.p.) once per day at 08:00 for 14 days. Rats were then housed in their home cages to observe spontaneous cocaine withdrawal signs 3, 10, and 30 days after the last cocaine administration.

2.2.1. Open field task

Exploratory locomotor activity was assessed in the open field test. The apparatus consisted of a gray box that was open at the top. The dimensions of the box were 100 cm × 100 cm × 50 cm, and the floor was divided into 25 squares (20 cm × 20 cm). The arena was illuminated with a 40 W lamp at the center, 60 cm above the floor. Each rat was placed gently in the center square of the open field, and behavior was videotaped for 5 min. The measurements included the number of squares entered (all four paws inside the square), and number of rearings (i.e., the animal standing on its hind legs with forelegs in the air or against the wall). After each rat was tested, the box was thoroughly cleaned to remove odor cues.

2.2.2. Elevated plus maze

The elevated plus maze (EPM) consisted of two opposite open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm, surrounded by a 40 cm high black wall) elevated 75 cm from the floor. Individual trials lasted 15 min each. An entry into an arm was deemed to have occurred when all four paws and the base of the tail were inside the arm. At the beginning of each trial, animals were placed in the center of the maze, facing an open arm [30]. The maze was cleaned with a 75% (v/v) ethanol solution after each trial. The number of entries and time spent in the open arms were measured in addition to the number of entries and time spent in the closed arms. Open-arm exploration was measured by normalizing open-arm entries to total entries (open arm + close arm) and open-arm time to total time (time spent in open arms + time spent in closed arms) [30]. In this paradigm, anxiety-like behavior is reflected by decreased open-arm exploration [31].

2.2.3. Sucrose preference test

The sucrose preference test was conducted as previously described [32–34]. Briefly, animals were habituated for 48 h to 1% sucrose (Sigma). Following a 4 h deprivation period, their preference for sucrose (1%) or water (identical bottles) was determined for 1 h.

2.3. Tissue sample preparation

The procedure was based on one used previously in our laboratory [35,36]. Rats were decapitated without anesthesia after the experiment. After decapitation, the brains were quickly extracted and frozen in −60 °C *N*-hexane and then transferred to a −80 °C freezer. We used a freezing cryostat (−20 °C; Reichert-Jung 2800 Frigocut E) to make 1 mm thick coronal sections. Bilateral tissue punches (12 gauge) of the SCN (located approximately −0.92 mm from bregma), prefrontal cortex (PFC; 16 gauge; located approximately 4.2 mm from bregma), ventral tegmental area (VTA; 16 gauge; located approximately −5.2 mm from bregma), and NAc (16 gauge for two punches; located approximately 2.2 mm from bregma) were then taken. Tissue punches were homogenized (10–15 s × 3, 5 s interval) with an electrical disperser (Wiggenhauser Sdn Bhd) after 30 min in RIPA lysis buffer (Beyotime Biotechnology, China; 20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3VO_4, 0.5 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride). Tissue homogenates were then subjected to 10,000 × *g* centrifugation at 4 °C for 5 min. All of the above procedures were performed at low temperature (0–4 °C). The protein concentrations of all samples were determined using a bichinconinic acid assay (Beyotime Biotechnology). Samples were further diluted in RIPA lysis buffer to equalize protein concentrations.

2.4. Western blot assays

The samples were treated according to our previous studies [35,36] with modifications. 4× loading buffer (16% glycerol, 20% mercaptoethanol, 2% sodium dodecyl sulfate [SDS], and 0.05% bromophenol blue) was added to each sample (3:1, sample:loading buffer) before boiling for 3 min. Samples were cooled and subjected to SDS-polyacrylamide gel electrophoresis (10% acrylamide/0.27% *N*,*N*′-methylenebisacryalamide resolving gel) for approximately 30 min at 80 V in stacking gel and approximately 90 min at 120 V in resolving gel. For each electrophoresis, increasing amounts of protein pooled from all samples were electrophoresed to produce a standard curve. Proteins were transferred electrophoretically to Immobilon-P transfer membranes (Millipore, Bedford, MA, USA) at 0.25 A for 2.5 h. Membranes were washed with TBST (Tris-buffered saline plus 0.05% Tween-20, pH 7.4) and then dipped in blocking buffer (5% bovine serum albumin [BSA] in TBST) at room temperature on an orbital shaker for 2 h. Membranes were then incubated overnight with anti-phospho-GSK-3β (1:1000; Cell Signaling Technology, Danvers, MA, USA) and anti-total-GSK (1:5000; Cell Signaling Technology) antibody in TBST plus 5% BSA and 0.05% sodium azide at 4 °C. The next day, after four 6 min washes in TBST buffer, the blots were incubated for 45 min at room temperature on a shaker with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG; goat anti-mouse IgG; Santa Cruz PI-1000; Vector Labs, Burlingame, CA, USA) diluted 1:5000 in blocking buffer. The blots were then washed four times for 6 min each in TBST and then incubated with a layer of Super Signal Enhanced Chemiluminescence substrate (Detection Reagents 1 and 2 at a 1:1 ratio; Pierce Biotechnology, Rockford, IL, USA) for 1 min at room temperature. The blots were wrapped with a clean piece of plastic wrap (no bubbles between blot and wrap) after dripping off excess mixture and then exposed to X-ray film (Eastman Kodak Company) for 30–300 s. Band intensities for GSK-3β and phosphorylated GSK-3β (pGSK-3β) were quantified using Quantity One version 4.4.0 software (Biorad Corporation, Hercules, CA, USA). Band intensities from each test sample were compared with the band intensities from the standard curves. The amount of the protein of interest in each sample was interpolated from the standard curve.