Behavioural Brain Research 243 (2013) 255–260

Contents lists available at SciVerse ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

The *mPer2* clock gene modulates cocaine actions in the mouse circadian system

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HIGHLIGHTS

- Cocaine acts in the SCN clock as a non-photic circadian entrainment stimulus.
- Mice with non-functional *Per2* are hypersensitive to non-photic actions of cocaine.
- Cocaine blocks the circadian phase-delaying action of light at night.
- Cocaine attenuation of photic phase-delays is heightened in *Per2* mutant mice.
- *Per2* is a potent (negative) modulator of cocaine's actions in the circadian system.

ARTICLE INFO

Article history:
Received 6 December 2012
Received in revised form 8 January 2013
Accepted 12 January 2013
Available online 17 January 2013

Keywords:
Cocaine
Circadian
Clock gene
Suprachiasmatic nucleus
Entrainment
Mouse

ABSTRACT

Cocaine is a potent disruptor of photic and non-photic pathways for circadian entrainment of the master circadian clock of the suprachiasmatic nucleus (SCN). These actions of cocaine likely involve its modulation of molecular (clock gene) components for SCN clock timekeeping. At present, however, the physiological basis of such an interaction is unclear. To address this question, we compared photic and non-photic phase-resetting responses between wild-type (WT) and *Per2* mutant mice expressing nonfunctional PER2 protein to systemic and intra-SCN cocaine administrations. In the systemic trials, cocaine was administered i.p. (20 mg/kg) either at midday or prior to a light pulse in the early night to assess its non-photic and photic behavioral phase-resetting actions, respectively. In the intra-SCN trial, cocaine was administered by reverse microdialysis at midday to determine if the SCN is a direct target for its non-photic phase-resetting action. Non-photic phase-advancing responses to i.p. cocaine at midday were significantly (~3.5-fold) greater in *Per2* mutants than WTs. However, the phase-advancing action of intra-SCN cocaine perfusion at midday did not differ between genotypes. In the light pulse trial, *Per2* mutants exhibited larger photic phase-delays than did WTs, and the attenuating action of cocaine on this response was proportionately larger than in WTs. These data indicate that the *Per2* clock gene is a potent modulator of cocaine's actions in the circadian system. With regard to non-photic phase-resetting, the SCN is confirmed as a direct target of cocaine action; however, *Per2* modulation of this effect likely occurs outside of the SCN.

1. Introduction

Cocaine abuse is associated with disturbances in the daily patterns of circadian-timed homeostatic functions, including those of the endocrine, autonomic, and immune systems [1,2]. These actions indicate that cocaine may directly or indirectly disrupt circadian timing, which could contribute to the process of addiction. The basis for such effects of cocaine may be related to the reciprocal interaction between cocaine and circadian clock gene activity. Cocaine is known to affect central clock gene expression, and conversely, circadian clock genes (in particular *Per2*) regulate cocaine intake and reward. The *Per2* clock gene is rhythmically expressed in the SCN, as well as in other regions, and stabilizes endogenous circadian pacemaker activity, as shown in studies from *Per2* mutant mice that express inactive PER2 protein [3,4]. Notably, *Per2* mutant mice exhibit greater frequencies of drug (cocaine or ethanol) self-administration [5,6], higher levels of voluntary drug intake [7,8], and stronger conditioned and two-bottle free-choice preferences for drug solutions over water compared to wild-type mice [5–8]. Behavioral analyses have shown that *Per2* mutant mice also have differential locomotor reactions to acute and repeated presentations of rewards, including cocaine, compared to wild-type mice [5,6,9].

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0166-4328/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbr.2013.01.014

Recently, we have reported that acute and chronic modes of cocaine administration are highly disruptive to photic and non-photic circadian clock phase-resetting and entrainment [10,11]. Acute cocaine treatment at midday promotes large non-photic phase-advance shifts of locomotor rhythms, and similar drug treatment at night blocks photic phase-delay shifts [10]. Notably, chronic forced and free-choice regimens of oral cocaine administration significantly alter the intrinsic free-running period of the circadian clock, an effect which persists for months after cocaine withdrawal [11]. We have also demonstrated that the SCN is a direct target of cocaine's photic and non-photic phase-resetting action *in vitro* and that this action is mediated by its inhibition of the serotonin transporter [10,12].

While the effects of the *Per2* mutation on drug intake and reward are well documented, data are lacking on whether this mutation affects circadian clock photic and non-photic responses to cocaine. By inference, such action would earmark the *Per2* gene as an important modulator of cocaine action in the circadian system. This information would significantly extend current knowledge of the genetics of drug addiction, particularly with regard to recent studies demonstrating that polymorphisms of *Per2* in humans are strongly tied to increased risk for cocaine dependence [13].

2. Methods

2.1. Animals

Male mice with a nonfunctional homolog of *Per2* (*Per2* mutant; B6.129S7-*Per2*^{tm1Brd}/J) and wild-type (WT; B6(Cg)-*Tyr*$^{c-2J}$/J) controls were raised from breeder pairs backcrossed on a C57BL/6 background (The Jackson Laboratory, Bar Harbor, ME, USA). Mice were 8-10 weeks old at the time of experimentation. Animals were singly housed in polycarbonate cages under a 12L:12D photocycle (270 lx; LD) in a temperature-controlled vivarium (23 °C). Food and water were *provided ad libitum*. All experiments were performed using the *National Institutes of Health Guidelines for the Care and Use of Laboratory Animals* and approved by the Kent State University Institutional Animal Care and Use Committee.

2.2. Behavioral measures

General circadian locomotor activity was recorded using overhead infrared motion detectors interfaced with a computerized data acquisition system (Clocklab; Coulbourn Instruments, Whitehall, PA, USA). Data were collected in 10 min bins. Activity onset associated with lights-off under LD (designated as zeitgeber time [ZT] 12) was defined by the initial 10 min period that: (1) coincided with an intensity of activity that exceeded 10% of the maximum rate for the day; (2) preceded by at least 4 hr of activity quiescence; and (3) followed by at least 60 min of sustained activity. Following release to constant darkness (DD) to examine phase-shifts using a modified Aschoff type II procedure [14] and rhythm period in response to drug administration and genotype, activity onset was used as the reference point for the beginning of the subjective night (designated as circadian time [CT] 12). Phase-shifts were calculated from the (1) back extrapolation of the least squares line through activity onsets on days 3–10 after treatment; and (2) extrapolation of the least squares line calculated from activity onset data collected for a minimum of 7 days prior to treatment. Assessments of changes in activity (duration and intensity) after i.p. and intra-SCN drug treatment were undertaken using data exported from a Clocklab data acquisition system. A count represented an individual event registered by an overhead infrared sensor. Activity duration represented the length of increased activity bouts (relative to pre-treatment level) immediately following treatment at midday. Each bout was defined as the sum of activity events collected in each 10 min bin.

2.3. Drug administration

In the systemic cocaine trials, mice received an intraperitoneal (i.p.) injection of physiological saline or cocaine hydrochloride (20 mg/kg; Sigma–Aldrich Corp St. Louis, MO, USA) dissolved in physiological saline. In the intra-SCN trial, artificial cerebrospinal fluid (ACSF) or cocaine hydrochloride (0.5 mM) dissolved in ACSF was perfused at a flow rate of 1.0 μl/min through a microdialysis probe (see below) targeting the lateral margin of the suprachiasmatic nucleus (SCN; anteroposterior, −0.46 mm from bregma; lateral, +0.20 mm from midline; horizontal, 5.50 mm from dura). Concentrically designed microdialysis probes were constructed from a 26-gauge stainless steel outer cannula into which was inserted 32-gauge fused silica tubing. Hemicellulose dialysis tubing (230 μm outer diameter; 12 kDa MW cutoff; SpectraPore, Fisher Scientific) was affixed to the outer cannula with epoxy glue. The active dialysis length was 1.0 mm. Theoretical probe efficiency was estimated *in vitro* by measuring the yield of cocaine from probes immersed in a 0.5 mM cocaine solution at 37 °C. Probe efficiency averaged ∼23%. Based on *in vitro* probe efficiency of 23%, this provided a theoretical cocaine concentration of ∼115 μM outside the probe. The microdialysis probe was inserted three days prior to intra-SCN drug administration which is sufficient for blood brain barrier repair and return of locomotor activity rhythms to baseline levels [see ref. [10]]. For probe implantation, animals were anesthetized with pentobarbital sodium (Nembutal; 35 mg/kg) and pretreated with Marcaine (0.25% bupivicaine; 0.05 ml) in the scalp area and atropine subcutaneously (0.09%; 0.10 ml) to reduce localized pain and respiratory occlusion, respectively. Probes were secured to the skull with dental cement and stainless steel screws. Surgery was undertaken at the beginning of the light phase under LD. Mice were returned to their home cages following surgery. Following experimentation, microdialysis probe placement was verified from 60 μm-thick cryostat sections stained with cresyl violet.

2.4. Experimental protocols

In all experiments, general locomotor activity rhythms of WT and *Per2* mutant mice were monitored for 2 wk under LD prior to experimentation to establish baseline conditions.

2.4.1. Experiments1 and 2

Experiments1 and 2 compared non-photic phase-advancing responses of *Per2* mutant and WT mice to i.p. and intra-SCN administration of cocaine at ZT 6 (n = 6/group), respectively. For the latter trial, animals were surgically outfitted with a microdialysis probe stereotaxically aimed at the lateral margin of the SCN three days prior to experimentation as described above. On the day of experimentation, microdialysis probes were continuously perfused with ACSF alone or ACSF + cocaine from a syringe pump (n = 4/group). Continuous 80 min perfusion of ACSF or ACSF + cocaine extended from ZT 6–7.3. In both trials, animals were released into DD at the onset of perfusion to assess phase-shifting. Immediately after treatment, the mice were released to DD for 2 wk to assess phase-shifting responses using the Aschoff type II procedure.

2.4.2. Experiment 3

Experiment 3 compared the action of i.p. administration of cocaine delivered 15 min prior to a phase-delaying 30 min light pulse (25 lx) or no light pulse beginning at ZT 16 (n = 6/group) between *Per2* mutant and WT mice. Immediately after the light pulse, the mice were released to DD to assess phase-shifting.

2.5. Statistical analyses

Univariate ANOVAs and subsequent Student Newman–Keuls *post hoc* mean comparison tests were used for treatment (cocaine or saline) and genotype (WT or *Per2* mutant) statistical comparisons of phase-resetting responses to cocaine administration. All statistical analyses were completed with SPSS 19.0 (Chicago, IL). The significance level was set at $p < 0.05$, in all cases.

3. Results

3.1. Experiments 1 and 2

3.1.1. Per2 mutation and cocaine-induced phase-resetting

Phase-advances of locomotor activity rhythms were significantly larger for both WT and *Per2* mutant mice treated with i.p. cocaine compared with saline or no injection ($F_{2,30} = 9.8$; $p < 0.01$; Fig. 1). There was a main effect for genotype, such that *Per2* mutants treated with cocaine had significantly (∼3.5 fold) larger phase-advance shifts compared with WTs (2.5 ± 0.7 h *vs.* 0.7 ± 0.3 h; $F_{1,30} = 16.5$; $p < 0.01$). There was also a significant interaction for genotype and treatment ($F_{1,30} = 10.6$; $p < 0.01$). Analyses of rhythm period under free-running (DD) conditions revealed a main effect for genotype (*Per2* mutant: 23.2 ± 0.3 h; WT: 23.9 ± 0.1 h; $F_{1,30} = 15.5$; $p < 0.01$), but not for treatment ($p > 0.05$). Within-genotype analyses revealed no differences in behavioral activity measures between i.p. cocaine and saline groups in *Per2* mutants (activity duration: 144 ± 55 min *vs.* 115 ± 38 min, respectively; total activity counts: 243 ± 95 *vs.* 496 ± 221, respectively; Student's t test for both measures; $p > 0.05$ for both). In contrast, WTs were more behaviorally reactive to i.p. cocaine compared with saline (activity duration: 210 ± 30 min *vs.* 90 ± 37 min, respectively; total activity counts: 432 ± 60 *vs.* 179 ± 84, respectively; Student's t test for both measures; $p < 0.05$ for both; Fig. 2).