Circadian Alteration in Neurobiology During 30 Days of Abstinence in Heroin Users

Su-xia Li, Jie Shi, David H. Epstein, Xi Wang, Xiao-li Zhang, Yan-ping Bao, Dai Zhang, Xiang-yang Zhang, Thomas R. Kosten, and Lin Lu

Background: Previous studies have shown that individuals withdrawn from chronic opiate administration undergo substantial elevations of cortisol levels with blunted corticotropin (ACTH) rhythms and that these changes persist beyond the 7–10 days of acute withdrawal symptoms. However, there are no published studies of changes in expression of clock genes or of other neuropeptides related to circadian-rhythm regulation, which may influence relapse susceptibility.

Methods: Blood samples were collected from 8 healthy control subjects and 16 heroin addicts during pharmacologically unassisted withdrawal on the 3rd, 10th, and 30th days of abstinence at 3-hour intervals for 24 hours. Outcome measures were the relative expression of clock gene mRNA (*hperiod1, hperiod2, hclock*) and the levels of serum cortisol, plasma ACTH, β-endorphin (β-EP), leptin, neuropeptide Y, interleukin-2 (IL-2), and tumor necrosis factor (TNF) in these subjects.

Results: Compared with healthy volunteers, abstinent addicts showed disruptions in diurnal rhythms of *hPER1* and *hPER*2 mRNA expression, along with disruptions in diurnal rhythms of cortisol, ACTH, β-endorphin, leptin, and IL-2 release. Several of these disruptions (*hPER1*, *hPER2*, ACTH, β-endorphin, and IL-2) persisted for the 30-day testing period, as did elevation of 24-hour levels of cortisol and decreases in 24-hour IL-2 and TNF levels.

Conclusions: These prolonged neurobiological changes may play a role in protracted opiate withdrawal symptoms and contribute to relapse vulnerability.

Key Words: Circadian rhythms, clock genes, cytokines, heroin addiction, hormones, peptides, spontaneous withdrawal

In humans and laboratory animals, resumption of drug seeking can be induced after extended periods of abstinence by reexposure to the drug itself (1,2) or by exposure to drug-related cues (3,4) and stress (5–8). A previous study reported the average time to relapse to drug use was 25 days, and the rate of relapse was 71% within the first 6 weeks and 95% within 3 months (9). Recent studies suggest that the high rate of relapse to opiate abuse in this time frame is closely related to prolonged vulnerability to craving, sleep disturbances, and negative affective states (10,11), a cluster of symptoms sometimes called the protracted withdrawal syndrome (12).

Circadian rhythms of eating and sleeping are also disturbed during these 6–12 weeks. Early studies showed that heroin produces a dose-related decrease in total sleep and sleep efficiency (13,14), but few studies have examined how opiate withdrawal is accompanied by disruption of circadian rhythms.

Circadian rhythms are partly controlled by "clock genes," which are expressed in the suprachiasmatic nucleus (SCN) of the hypothalamus, other brain regions, and peripheral tissues. The first clock gene discovered was *PER*, in *Drosophila* (15); *PER* has since been shown to have homologs in mice (e.g., *mPER1* and *mPER2*) and humans (e.g., *hPER1* and *hPER2*) (16). Other mammalian clock genes include *mCLOCK*/*hCLOCK* (in mice and humans, respectively); the functional importance of *hCLOCK* is supported by the finding that an *hCLOCK* polymorphism is associated with an innate preference for early or late rising in the morning (17).

To assess circadian rhythms of expression of *hPER1*, *hPER2*, and *hCLOCK* in humans undergoing heroin withdrawal (and healthy control subjects), we used peripheral blood mononuclear cells (PBMCs), which express clock genes in an oscillatory way and may therefore be useful for the investigation of human circadian rhythms and their disruption (18). In the same humans, we also examined circadian rhythms in blood levels of cortisol and six peptides: corticotropin (ACTH), β-endorphin, leptin, neuropeptide Y (NPY), interleukin-2 (IL-2), and tumor necrosis factor (TNF). We examined these markers because cortisol levels are known to be increased during heroin withdrawal (19–21), and its release follows a well-known circadian pattern. Long-term opioid consumption appears to cause feedback inhibition of the ACTH/β-endorphin system, leading to decreases in plasma levels of ACTH (22–24), although some authors have observed elevated ACTH (25) and circadian patterns of ACTH release that are as same as cortisol (26). NPY release in healthy adults follows a circaoctohoran pattern (27), which is implicated in circadian rhythms, feeding, anxiety, and reward-related behavior (28). Plasma leptin concentrations undergo nonrandom fluctuations over 24 hours, with an underlying 24-hour periodicity (29–31), and it is involved in food-deprivation-induced reinstatement of heroin seeking in rats (32). Opiates suppress immune function through brain pathways (33) that activate the hypothalamic-pituitary-adrenal (HPA) axis (34) and the sympathetic nervous system (35,36). Both IL-2 and TNF release show circadian variation (37).

Most of the peptides we measured can enter the brain through various processes (38–45). For example, NPY can readily diffuse across the blood-brain barrier (BBB) (40), and IL-2 and TNF-α

From the National Institute on Drug Dependence (S-xL, JS, XW, X-lZ, Y-pB, LL), Peking University, Beijing, China; Intramural Research Program (DHE), National Institute on Drug Abuse, National Institutes of Health, Baltimore, Maryland; Institute of Mental Health (DZ), Peking University, Beijing, China; Division of Alcohol and Addictive Disorders (X-yZ, TRK), Baylor College of Medicine, Houston, Texas.

Address reprint requests to Lin Lu, M.D., Ph.D., National Institute on Drug Dependence Peking University, 38, Xue Yuan Road, Haidian District, Beijing 100083, China; E-mail: linlu@bjmu.edu.cn.

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can enter the brain by nonsaturable (38,42) and saturable processes (41), respectively. Although β-endorphin cannot cross the BBB, there is a significant linear correlation between plasma and cerebrospinal fluid (CSF) β-endorphin levels (46). As for the expression of clock genes, we have conducted an animal experiment to determine whether the gene expression in PBMCs is paralleled by similar changes in the hypothalamus.

Methods and Materials

Subjects

Twenty male heroin-dependent individuals from 20 to 37 years old (mean ± SD: 30.11 ± 4.17 years) who met the DSM-IV criteria for opioid dependence were recruited from the Drug Detoxification and Rehabilitation Center of Kunming, Yunnan, China. Participants completed a pharmacologically unassisted heroin detoxification and remained free from opioids for 30 days after enrollment. The 16 heroin addicts who finished the study reported 90 (± 59) months of drug dependence, 5.5 ± 4.1 months of continuous drug use before enrollment, and a daily intake of heroin ranging from .4 to 2.5 g (mean ± SD: .8 ± .5 g). In addition to excluding adjunctive medications for withdrawal symptoms, the other exclusion criteria were as follows: 1) previous or current use of cocaine, methamphetamine, MDMA, or other illicit drugs; 2) diastolic blood pressure less than 60 mm Hg or heart rate less than 60 beats per minute; 3) HIV serostatus positive; 4) current or past serious physical illness (e.g., active tuberculosis, acute hepatitis or cirrhosis, renal illness, cardiovascular illness, or unstable diabetes); 5) immune disorders; and 6) major Axis I psychiatric disorders.

Control participants were eight physically and mentally healthy male volunteers recruited from the local community who were matched for age (mean ± SD: 29.0 ± 4) and monitored with urine drug screens. The following were exclusions: 1) any of the exclusion criteria for the experimental group, 2) past or present DSM-IV Axis I or Axis II disorders, 3) sleep disturbance, 4) smoking, 5) age less than 20 years, 6) use of any medication within the previous 30 days, 7) any current or past physical illness that would be aggravated or reappear if the individual participated in the study, and 8) positive breath alcohol or urine drug screen.

Potential participants who appeared to meet inclusion criteria after an initial interview proceeded through a sequence of in-depth screening procedures, including the Structured Clinical Interview for DSM-IV disorders (SCID), physical examination, electrocardiogram, and urine drug screening. All the research procedures had been approved by the ethics committee of the Peking University Health Science Center, Beijing, China, and each participant provided written informed consent before participating. Each participant was paid 200 RMB ($25) upon completion, and the opioid-dependent participants remained in the rehabilitation center after the study.

Rats

Adult male Sprague-Dawley rats were used for animal experiments (see Methods in Supplement 1 for additional information). Experiments were carried out in accordance with the Institutional Animal Care and Use Committee's recommendations at the University of Peking.

Experimental Procedure

All abstinent heroin addicts underwent three experimental sessions. The first session started after 35–72 hours of abstinence from heroin (mean ± SD: 47.74 ± 8.97), the second and the third session were on the 10th and the 30th day of abstinence. Each healthy volunteer underwent one experimental session.

Each participant arrived at the experimental ward before 9:00 AM on the first day and remained there until 3:00 PM the next day. At 9:00 AM on the first day, an intravenous catheter was inserted into a forearm vein. From 9:00 AM to 5:00 PM, the participant sat in a recliner to adapt to the experimental environment but was not allowed to sleep. At 5:00 PM, the first blood sample was drawn; eight blood samples were collected 5:00 PM, 8:00 PM, 11:00 PM, 2:00 AM, 5:00 AM, 8:00 AM, 11:00 AM, and 2:00 PM. Lights were turned off at 10:30 PM, and the participant was woken at 7:00–7:30 AM. The tubing system was kept patent by continuous infusion of heparinized isotonic saline between blood samplings. Participants received standardized meals and were allowed to watch television, read books, and talk to each other. Before the first blood sample was drawn in each experimental session (Days 3, 10, and 30), negative affect in heroin-addicted participants was assessed with the Hamilton Anxiety Scale (HAMA) and the Beck Depression Inventory (BDI), along with assessment of physical signs and symptoms.

For examination of the peripheral and central oscillation experiments, rats were killed every 3 hours during one 24-hour period (see Methods in Supplement 1 for additional information).

Hormone, Peptide, and Cytokine Assessment

Serum concentrations of cortisol and plasma concentrations of ACTH, β-endorphin, leptin, NPY, IL-2, and TNF were detected by a radioimmunoassay (RIA) kit (Furui, Beijing, China). The intraassay and interassay coefficients of variation were less than 5% and 10% for cortisol, leptin, and TNF; less than 7.7% and 9.5% for NPY; less than 6% and 10 % for ACTH and β-endorphin; and less than 7.0% and 10% for IL-2.

RNA Isolation and Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, California) for the human and rat PBMCs and RNAsimple Total RNA Kit (Tiangen Biotech, Beijing, China) for rat hypothalamic tissues according to the manufacturer's specifications. We carried out cDNA and a quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) to assess the level of *hPER1*, *hPER2*, and *hCLOCK* expression relative to expression of the housekeeping gene *GAPDH* on samples from healthy control subjects and a randomly selected sample of 8 of the 16 abstinent subjects and all the rats (see Supplement 1).

Data Analyses

Participant demographics were characterized using descriptive statistics (means and SDs). Relative expression of *hPER1, hPER2,* and *hCLOCK,* and levels of serum cortisol and plasma ACTH, β-EP, leptin, NPY, IL-2, and TNF, were analyzed using SAS 8.0 PROC analysis of variance (ANOVA) of the eight serial assessments over 24 hours. For abstinent addicts, the ANOVAs had two repeated factors, Day (3, 10, and 30) and Time of Day; for healthy volunteers, the ANOVAs had only one repeated factor, Time of Day. For each of the 10 dependent variables, differences between groups were also assessed in three separate ANOVAs comparing the healthy volunteers to the abstinent addicts on each of the abstinent addicts' 3 test days. The circadian rhythms of each of the 10 dependent variables were assessed with the single-cosinor method. We calculated the mesor (middle value of the fitted cosine, representing a rhythm-adjusted mean) and the amplitude (half the difference between the minimum

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