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Sleep Deprivation in the Rat: II. Methodology

Bernard M. Bergmann, Clete A. Kushida, Carol A. Everson, Marcia A. Gilliland, William Obermeyer, and Allan Rechtschaffen

Sleep Research Laboratory, Departments of Psychiatry and Behavioral Sciences, University of Chicago, Chicago, Illinois, U.S.A.

Summary: Methods common to several studies in this series are described. A key feature is a sleep deprivation apparatus in which an experimental and a yoked control rat are housed on opposite sides of a divided disk suspended over shallow water. When the experimental rat enters a "forbidden" sleep stage, the disk is automatically rotated, forcing the experimental rat to walk to avoid being carried into the water. The control rat receives the same physical stimulation but can sleep ad lib when the disk is stationary. **Key Words:** Sleep deprivation—Sleep deprivation methods—Sleep deprivation apparatus.

This article defines the terms and describes the methods that are common to several of the studies on sleep deprivation in the rat in this series.

FREQUENTLY USED TERMS AND THEIR ABBREVIATIONS

These are frequently used terms and their abbreviations found in this series: wakefulness (W); total sleep (TS); paradoxical sleep (PS), low amplitude electroencephalogram (EEG) and electromyogram (EMG), and high amplitude theta activity; non-rapid eye movement (NREM) sleep, all sleep which is not PS; low-amplitude sleep (LS), NREM sleep with low amplitude EEG, EMG, and theta (1); high EEG amplitude sleep (HS), NREM sleep with high EEG amplitude; HS1, the fraction of HS with EEG amplitude below the HS modal EEG amplitude; HS2, the fraction of HS with EEG amplitudes above the HS mode. Typical values for rats housed in cages without disks were 43% W, 27% HS1, 18% HS2, 8% PS, and 4% LS (2).

Total sleep deprivation (TSD) are procedures or rats in which the total deprivation of sleep is the goal; total sleep control (TSC) are yoked controls for TSD. Other terms that are frequently used are the following: paradoxical sleep deprivation (PSD); controls for PSD (PSC); HS2 deprivation (HS2D); controls for HS2D (HS2C). A "run" refers to all experimental procedures on one deprived rat and its yoked control.

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Address correspondence and reprint requests to Dr. A. Rechtschaffen at the Sleep Research Laboratory, 5743 S. Drexel Avenue, Chicago, IL 60637, U.S.A.

SUBJECTS

Subjects were male Sprague Dawley rats, approximately 4 to 6 months old and initially weighing 425–525 g. From their arrival at the laboratory, they were maintained in constant light to flatten their circadian rhythms (3) as a precaution against the possibility of confounding the effects of sleep loss with effects of shifts in phase, period, or amplitude of circadian rhythms.

SURGICAL PROCEDURES

Rats were given ketamine hydrochloride and xylazine as required to maintain deep anesthesia. To record cortical EEG with a minimum of theta activity, two miniature screw electrodes were placed through the skull ipsilaterally in a lateral position: 1 mm posterior to bregma, 3 mm lateral to the central suture; and 1 mm anterior to lambda, 4 mm lateral to the central suture. To maximize theta activity in cortical recordings, two screws were placed ipsilaterally in a more medial position: 3 mm anterior to bregma, 1 mm lateral to the central suture; and 4 mm anterior to lambda, 1 mm lateral to the central suture. Temporalis EMG electrodes were implanted by the method of Rosenberg et al. (4). PSD and PSC rats were also implanted with silver plates in the nuchal muscles and with pontine spike electrodes according to the description of Farber et al. (5). In some rats, wires were positioned on either side of the rib cage and sutured bilaterally to the cutaneous maximus muscles to record heart rate.

Electrode wires were soldered to miniature plugs fastened to the skull. Several procedures were used to maximize adhesion of the electrode plug to the skull for extended periods—sometimes longer than two months: (a) The skull was cleansed with anhydrous ether. (b) To strengthen the skull, it was treated with an acidulated fluoride phosphate solution (6). (c) The skull was etched with Scotchbond Etching Gel (3M Dental Products, Catalog No. 7423). (d) A grid of 3-0 surgical steel wire was laced across the skull surface through small holes drilled into the dorsolateral flexures to serve as a support for a subsequently applied ceramic base (7). (e) The skull was covered with a thin layer of P-10 Resin Bonded Ceramic (3M Dental Products, Catalog No. 9301S). (f) The recording plug was cemented to the ceramic base with a lightweight dental acrylic (Minit Weld, Teledyne Getz). (g) Small "cliffs" were etched into anterior and posterior portions of the skull not covered with ceramic to increase the gripping surface for the acrylic.

A temperature sensitive transmitter (Mini-Mitter, Model M, Mini-Mitter Co., Sunriver, OR) was placed in the peritoneal cavity. To prevent the tips of the tails from becoming pinched in the 2-mm slit where the platform of the deprivation apparatus rotates beneath the cage wall, the last 1.5 cm of tail was amputated.

To permit daily blood sampling, most TSD, TSC, PSD, and PSC rats were implanted through the external jugular vein with a catheter positioned into the right atrium of the heart. The catheter was formed from a 45-cm strand of pliable, nontoxic, nonthrombogenic, polyurethane tubing, 0.050 in o.d. × 0.025 i.d., (Micro-Renathane-050, Braintree Scientific). Small bulges were formed at 3.4 and 3.6 cm from one end of the tube either by heating and then compressing it or by fitting two 1-mm sections of tubing (from Miniset Vein Infusion Set, 1.1 mm i.d. × 2.1 mm o.d., Travenol Industries) over the catheter to form outer rings 1 mm apart. A 1.5-cm incision was made in the right ventral neck region, and the external jugular vein was isolated and ligated approximately 1 mm posterior to the junction of the vein with its posterior branch. A section

of 4-0 suture was placed under the jugular approximately 2 mm anterior to its junction with the cephalic vein. The untreated end of the catheter was inserted into the jugular 1 mm anterior to the thread and gently pushed through the vein to a depth of 3.4 cm, which, for the size and strain of rats we used, positioned its blunt cut tip in the right atrium and one of the preformed bulges just inside the vein. The suture was then tied around the vein between the bulges to prevent slippage. Two additional sutures were used to anchor the catheter subcutaneously. The catheter was drawn subcutaneously along the neck to the posterior part of the skull incision. There it was cemented to the head plug and extended up the recording cable where it was capped with an injection port. After patency was checked, 0.25 ml of heparinized saline was injected, followed by a 1.0-ml injection of sterile saline to restore blood volume. The catheter was then filled with an additional 0.25 ml of heparinized saline.

EXPERIMENTAL SCHEDULE

Rats resided in the experimental apparatus for at least 7 days of adaptation, during which they rested on a solid floor that was installed over the apparatus disk and covered the entire bottom of the cage. Then, rats began a 2-week baseline period during which the "adaptation" floor was removed and they resided on the apparatus disk (over water) which was rotated once an hour for 6 s to wipe boli from the disk surface and to familiarize the rats with disk rotation. All variables were measured during baseline. Typically, sleep and stage percentages were slightly lower during baseline in the apparatus than in "standard" recording cages.

EXPERIMENTAL APPARATUS AND DEPRIVATION PROCEDURES

The deprivation apparatus (Fig. 1) has been previously described (8). A pair of clear, smooth plastic cages, each 60 cm (l) × 20 cm (w) × 60 cm (h), house the experimental

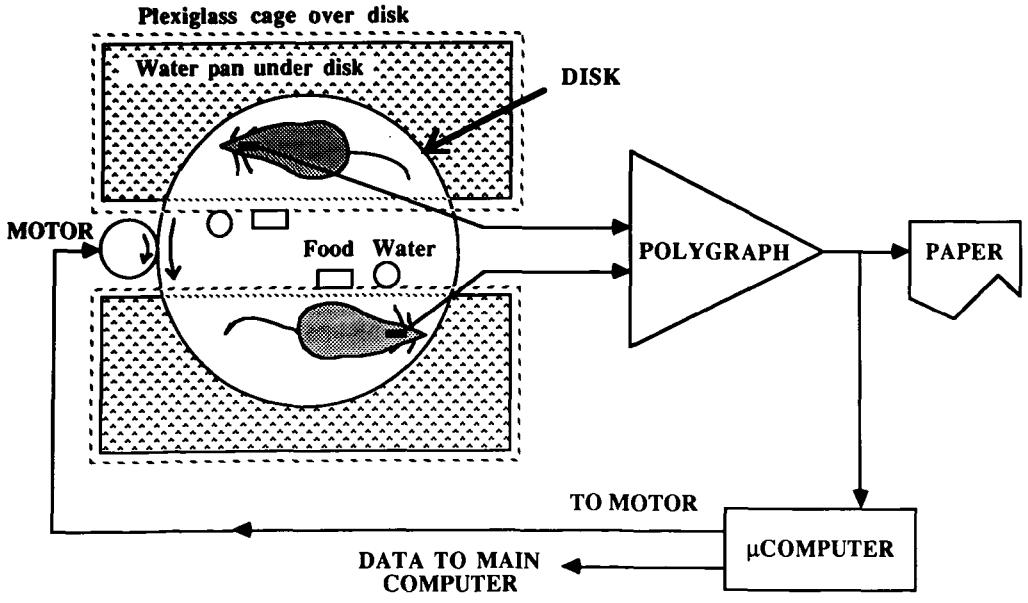

FIG. 1. Schematic diagram of the sleep deprivation apparatus.

rat and its yoked control. A 46-cm diameter smooth plastic disk, with its center in an alley between the cages, protrudes 15.5 cm under each cage to provide a partial floor with approximately the same area as a home cage (492 cm^2). Beneath each side of the disk and extending beyond it to the walls of each cage is a tray of 2–3 cm deep water. Cage temperature was regulated at 28–29°C (rat thermoneutral) by infrared lamps; pan water was 3–4°C lower. Drinking water was available ad lib from bottles. Food was available ad lib from feeders specially designed to minimize waste.

Each rat was connected by a recording cable to a commutator suspended from a counterbalanced boom, permitting free movement throughout the cage. Signals passed to a polygraph in an adjoining room for continuous ink recording and then to an AIM-65 microcomputer programmed to rotate the disk in a randomly chosen direction at a rate of 3.33 r/min whenever an experimental rat entered a "forbidden sleep stage." Typically, rats awakened when the disk started to rotate and walked opposite to disk rotation to avoid being carried into the water. When the signals indicated that the rat had not been in the forbidden state for about 6 s, rotation was ended.

A PDP 11-23 provided each AIM-65 with timing signals for 30-s epochs. For TSD, the disk was rotated whenever (a) the 2-s floating average EMG amplitude fell below criterion, thereby indicating the start of LS or PS; (b) with low to moderate EMG, instantaneous EEG amplitude rose above criterion, thereby indicating the start of HS; (c) with low EMG, the one-half or full epoch cumulated theta was above a "moderately high" criterion, thereby indicating the start of PS; or (d) with low EMG, the 2-s floating average theta rose above a "high theta" criterion (set high to avoid triggering during wakefulness), thereby indicating the start of PS. This criterion was a back-up to detect PS episodes not caught by condition (c).

For PSD, the disk was rotated if the 2-s floating average EMG and 2-s floating average EEG were both below criterion while pontine spike levels were above criterion, or if the one epoch cumulated theta was above criterion while EMG was below criterion. The latter option was a back-up in case pontine recordings failed. For HS2D, the disk was rotated when the 2-s floating average EMG was low to moderate and the 2-s floating average EEG exceeded a criterion corresponding roughly to the modal EEG amplitude during baseline HS sleep.

Criterion selection was guided initially by baseline values and subsequently by on-line polygraph tracings, behavioral observation, and scored sleep during the previous 24 h. Criterion levels always reflected a compromise between maximizing deprivation and minimizing unnecessary disk rotation.

For partial TS, PS, or HS2 deprivation, the AIM-65 was additionally programmed to recognize and count 30-s epochs of the restricted stages so that in a given time block (usually 4 h), a rat was allowed a given quota of TS, PS, or HS2 and, when the quota was exceeded, the deprivation program was instituted for the remainder of the block. Any unused portion of the quota was added to the quota for the next time block.

Each 30 s, data for the preceding epoch, including the integrated, rectified EEG, EMG, and theta values for both experimental and control rats, were passed from the AIM-65 to the PDP 11-23 for storage. Every 24 h, the data were computer scored for sleep stages using the PASS scoring system, which has been validated against visual and behavioral scoring (1). Some adjustments specific to the deprivation studies were necessary to detect short sleep fragments within 30-s epochs. Computer scores were regularly compared to the polygraph record, and, if they failed to match well, the data were rescored either by the computer with revised criteria or manually.

Sleep deprivation depended on the rats' aversion to water. Rats rarely entered the water spontaneously. Our earlier attempts at using electrical, vestibular, or tactile stimulation to awaken sleep-deprived rats foundered as progressively more frequent and intense stimuli eventually became incapable of preventing target sleep stages. The disk method had the advantages of (a) mild stimulation to awaken the rat, (b) stimulation only when it was indicated by physiological recording, (c) delivery of the same physical stimulus to a yoked control rat, and (d) requiring only a simple, nontaxing response (awakening and walking a few steps) to stop stimulation.

IMMERSION CONTROLS

Yoking was imperfect, because, as sleep deprivation continued, deprived rats entered the pan water more frequently than controls. Most immersions were partial and occurred when the rat allowed disk rotation to carry its forepaws into the water while it pedalled backward with its hindpaws on the disk. Later in deprivation, rats sometimes entered the water fully when they failed to awaken with disk rotation or were unable to negotiate disk rotation, seemingly because of weakness or ataxia; these immersions were brief, as rats quickly returned to the disk. Since the pan water was only 2–3 cm deep, the upper half of the body was rarely immersed. Full immersions were relatively infrequent; TSD rats, which had the highest immersion rate, were immersed on less than 2% of disk rotations. Nevertheless, immersion control (IC) procedures seemed indicated.

From slow motion play back of 4–5-h videotape segments of sleep deprived rats recorded every 2–4 days, we determined the frequency and duration of partial immersions (2 or 3 paws in water) and "full" immersions (4 paws or torso in water). Later, IC rats were maintained on disks that closely resembled the deprivation disks, except that instead of rotating, they could be lowered into the water pan. IC rats were scheduled for automatic immersions at rates matched to individual sleep-deprived rats. Since the IC apparatus could deliver only full immersions, rate matching was based on two partial immersions in a deprived rat equals one full immersion in the IC rat. Because of mechanical limitations of the IC apparatus, immersion duration could not be matched. Immersion durations in IC rats averaged 2.4 times longer than in matched TSD rats and 5.1 times longer than in matched PSD rats.

OTHER VARIABLES MEASURED

Because of limited resources and the later addition of new variables, not all variables were measured in each rat. Number (n) of rats in each analysis will be given with the results.

Body weight, food intake, and water intake were measured daily. Water intake was underestimated by an unknown amount because rats occasionally drank the pan water even though the pan water of sleep-deprived and control rats had been laced with quinine (approximately 87 mg/L of pan water). It is doubtful that any of the pathological or thermal effects we observed resulted from the quinine. Similar pathological consequences of TSD were observed in our earlier study (8), in which quinine was not always used. Also, attribution of pathological effects to the use of quinine in the present series would require that sleep-deprived rats drink more pan water than control rats—a phenomenon we did not observe. Furthermore, the lowest lethal dose for quinine given subcutaneously to rats is 200 mg/kg (9). Given the dilution of quinine in the pan water,

rats would have had to absorb about 900 ml of pan water to approximate this dosage. Our rats typically drank 35–45 ml/day from the drinking bottles. Satinoff (10) found that 25 mg/kg of quinine HCl (i.p.) had no effect on body temperature at ambient temperatures higher than 23°C. Our rats were maintained at an ambient temperature of about 29°C. They would have had to drink an average of 115 ml of pan water to approximate the Satinoff dosage.

Daily energy expenditure (EE) was estimated by the following formula: EE = [caloric value of food taken from hopper × fraction of food energy metabolized] + caloric value of weight loss. Caloric value of food was 4.058 kcal/g as determined by bomb calorimetry. The fraction metabolized was estimated at 0.8 based upon bomb calorimetry of wastes in five runs as described in Part V of this series (11). To smooth erratic fluctuations, weight change was based on a 5-day, 1:2:4:2:1, weighted average. The conversion of weight change to energy was estimated at 7 kcal/g, based on the estimate that approximately three-fourths of weight loss was fat.

Appearance of rats was rated on a 6-point scale (1 = completely healthy, 6 = extremely debilitated) from slide photographs taken every 2–3 days. For each point, prior scaling produced two reference photographs on which there was near unanimous agreement by five judges. Then all photographs were randomized with respect to run, experimental condition, and time in experiment; this information was withheld from two independent judges who rated each of the photographs. Interrater reliability was $r = 0.82$ for PSD and PSC rats, and 0.86 for TSD and TSC rats. The average of the two raters was used as the appearance score.

Body temperature was measured at 5–13-h intervals at least three times daily. Temperature measurement was not continuous, because it required positioning of the radio receiver relative to the transmitter in the rat.

Starting on the third adaptation day, 1–1.5 cc of blood were drawn daily from cannulated rats as follows: (a) A 0.1-cc dose of heparin sodium (1,000 U.S.P. units/ml) was injected into the catheter. (b) After 2 min, the heparin was removed and blood was drawn at 1 cc/min. (c) Lost blood volume was replaced with an equivalent volume of heparinized saline [1.3 cc heparin sodium (1,000 U.S.P. units/ml), 18.2 cc sterile saline, 0.5 cc ampicillin (50 mg/ml)]. (d) Blood samples were either analyzed immediately (glucose and hematocrit); placed in prechilled collection tubes in ice and delivered for analysis, or centrifuged at 10,000 r/min for 10 min to separate the plasma that was stored frozen at −40°C for later assay. (e) Blood cells were mixed with heparin saline solution equal to the removed plasma and reinjected to prevent anemia. (f) The catheter was heparinized with a 0.6-cc "flush" of heparinized saline to clear residual blood from the catheter's lumen and to prevent clot formation during the 24 h between blood drawings. Assays were performed by standard procedures. Except for glucose levels, assays were always blind to group or day. Because sample volume was limited, blood parameters were evaluated on rotating schedules, which will be described with results. For glucose tolerance tests, whole blood glucose levels were measured with Dextrostix reagent strips No. 2895 (Miles Laboratories) and an Ames Glucometer Reflectance Photometer. Urine samples were collected by holding rats over a glass tray and were analyzed semiquantitatively with N-Multistix SG reagent strips (Miles Laboratories).

SURVIVAL AND NECROPSY

Survival time was defined as the number of days from the start of sleep deprivation to either the spontaneous death or the sacrifice of a rat when death seemed imminent.

In most experiments, the intent was to sacrifice animals within a day or two of probable death in order to obtain viable tissue samples. Imminent death was judged by the following morbid symptoms in rats that died in the first TSD study and that were not sacrificed "in time" in the present studies: (a) severely debilitated appearance; (b) marked decline from maximal food intake; (c) severe ataxia or weakness, sometimes indicated by difficulty in negotiating the moving disk; (d) precipitous decline in body temperature; (e) marked edema of the paws; and (f) loss of EEG amplitude. That potential survival probably was not underestimated by more than a day or two in sacrificed animals is indicated by the fact that, of the rats we intended to sacrifice, 27% died before they could be sacrificed, i.e., we overestimated survival time. Whenever a deprived rat was sacrificed, its yoked control was sacrificed within the half-hour. Sacrificed rats were exsanguinated under anesthesia by cardiac puncture. Internal organs were examined for visible pathology, and several were weighed.

DATA ANALYSIS AND PRESENTATION

On the assumption that a pathological syndrome develops progressively in sleep-deprived rats, but at different rates, and that the rats die when the syndrome reaches a certain point, survival time, rather than chronological time, was used as the time scale for analysis. Each rat's survival time was divided into quarters, and data were averaged for each quarterly bin, first within rats and then across rats. In a very few cases, data were interpolated or extrapolated to provide a data point for a particular bin for a given rat; this was always decided without knowledge of the values involved. These procedures permitted a balanced design analysis of covariance with time as the covariate. Although the analysis yielded effects for group (deprivation vs. control), run, time (quarters), run by time interaction, and group by time interaction, in most cases only the group by time interaction will be reported. Other effects were generally not directly relevant to major issues, whereas the group by time interaction evaluates the critical issue of whether there are progressive changes in the experimental rat compared to the yoked control. Statistical significance was defined by $p < 0.05$. To facilitate comparisons of change across variables and experiments, graphs are presented in terms of mean percentage changes of rats from their individual baseline values. In most graphs, deprived rats are represented by solid lines, controls by dashed lines, and vertical lines at data points indicate standard error. In the text, standard deviation is indicated by $\pm$. Correlations (r) are Pearson product-moment coefficients.

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