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The experience in the water maze task can affect the circadian rhythm of locomotor activity

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Abstract
Wistar rats maintained in cages with running wheels and submitted to a skeleton photoperiod or to a light – dark cycle were tested in the Morris water maze. Half of the animals were exposed to the task during their active phase while the other half was exposed during their inactive phase. The effect of the experience in the water maze, a strong arousing event, on the rhythm of wheel-running activity was evaluated. In the first experiment, a group of animals submitted to a skeleton photoperiod was trained every day in the reference memory version of the task. The novel experience in the water maze had a strong phase-dependent masking effect: it produced an intense post-training bout of activity in the animals tested during their inactive phase. Another experiment was run using single working memory sessions in the water maze and with animals submitted to a light – dark cycle. The circadian rhythm of locomotor activity was evaluated on undisturbed days and compared with testing days. The experience in the water maze produced a significant increase in variability of activity onset during both circadian phases. Taken together, the data suggest that there is a modulating effect of the arousing experience in the pool on the overt circadian rhythm of locomotor activity.

Keywords: *Non-photic stimuli, Morris water maze, Wistar rats, wheel-running activity*

Introduction

Rhythmicity is ubiquitous in most physiological and behavioral variables. In mammals, the circadian timing system is responsible for this temporal organization, the suprachiasmatic nuclei, localized in the anterior hypothalamus, being the core of this system (Moore 1983). The biological clock generates rhythms with a frequency close to, but not equal to, 24 hours. This system allows the temporal coordination of various physiological processes within the organism as well as the temporal coordination of the organism with the external world. In order to fulfill these functions these rhythms must be synchronized to the exact 24 h cycle of the physical world. The daily light – dark cycle has been considered to be the dominant cue used by organisms to synchronize their biological clocks to the environment (Zordan et al.

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2001). However, today it is known that behavioral events can also exert an important synchronizing action, or at least interact with the photic events, in order to determine the proper timing of the organism's functions (Mrosovsky 1996).

It is well known that situations involving physical activity and/or arousal are capable of resetting the phase of the circadian clock (Turek 1989; Mrosovsky et al. 1989; Mrosovsky 1996). Such inputs also affect phase angles of entrainment to a light–dark cycle and free-running periodicity; in addition, they are capable of producing after-effects and splitting-like states (Janik & Mrosovsky 1993; Janik et al. 1994; Stokkan et al. 2001). The idea that pacemakers can be reset by stimuli other than light has been considered for a long time (Mrosovsky et al. 1989; Turek 1989; Mrosovsky 1996). Many experiments, mainly in hamsters, show that arousing stimuli such as cage-change, social interaction or novelty-induced running exert measurable effects on circadian rhythms. A phase response curve for these non-photic inputs can be obtained just as for light. Generally, a behaviorally arousing event produces phase advances when it occurs during the mid- to late subjective day (a time when the hamster is normally inactive), while phase delays occur when the event takes place in the late subjective night and early subjective day (Mrosovsky et al. 1989). The effects of non-photic behavioral arousing events on pacemakers are of practical interest when studying learning processes. Most of the learning tasks used for rodents imply strong arousal/activation induced states. In addition, most laboratories test animals during the light phase, which corresponds to the inactive phase of rodents. Therefore, at the moment of testing there could be a major arousal-induced phase shift.

The Morris water maze task (Morris 1981) is one of the most popular behavioral models for investigating the neurobiological basis of spatial learning and memory. In this task, animals have to find a hidden platform 2 cm underneath the water surface in order to escape the water. In the variable-start-position version of this task, the rat is trained to reach the hidden platform departing from different starting points at the pool edge. Optimal performance requires knowledge of the relative positions of the multiple extra-maze cues and of the platform relative to these cues; this involves navigation based on spatial strategies. Behavioral tasks requiring reference memory emphasize relevant information applicable to all trials. According to this view, the hidden platform version of the water maze task may be considered a spatial reference memory task if the platform position is kept constant throughout all training sessions. Tasks requiring working memory emphasize relevant information applicable to a specific trial that does not apply to others. Thus, if a different position for the hidden platform is used on each day of training, the critical spatial information will apply only to that specific session; therefore, it is considered a working memory task (Morris & Frey 1997; Xavier et al. 1999).

The Morris water maze task certainly generates an intense arousal in the animals. This is due to the diverse and variable stimuli associated with the situation, such as the fact that they are forced to produce motor behavior (swimming) added to the stress-generating, and consequently arousing, characteristics of the task, particularly in the early stages of training. In the present study we examined if this general activation produced by the animals' experience in the water maze task affects the circadian rhythm of locomotor activity. Wistar rats maintained in cages with monitored running wheels and submitted to skeleton photoperiods or light–dark cycles were tested in the Morris water maze during their active and inactive phases.

General methods

Animals and apparatus

Sixty male Wistar rats, varying from 4 to 10 weeks old, were purchased from the Instituto de Ciências Biomédicas and Faculdade de Medicina Veterinaria e Zootecnia, Universidade de

São Paulo. Upon arrival, the animals were group housed (four per cage) in the animal facility under a 12:12h light:dark cycle (LD; lights on at 07:00h) and left 1 – 2 weeks for acclimatization. Temperature was maintained constant at 22 ± 3°C. Food and water were available *ad libitum*. Animals were distributed in three conditions (see below). In each condition, animals were divided into two equal groups; one group was tested during the active phase and the other during the inactive phase.

A round, black, fiberglass pool, 200 cm in diameter and 50 cm high, was filled to a depth of 25 cm with water (26 ± 1°C) rendered opaque by the addition of milk. A movable, transparent circular plastic platform 9 cm in diameter, mounted on a plastic column, was placed in the pool about 2 cm below the water surface. The platform location depended on the behavioral testing procedure (see below). A video camera positioned approximately 290 cm over the center of the pool was connected to a video tracking digitizing device (VP112, HVS Image Ltd., Hampton, UK) and to a PC computer system programmed to collect and analyze the animals' swim-paths.

The light intensity in the experimental room was 11 – 24 lux at the water level when testing animals in dim light. This was the minimum intensity required for the normal functioning of the recording system. This dim light was provided by indirect filament lamps connected to dimmers (Alumbra 1000 W). When testing animals during the light phase of the LD cycle, normal fluorescent light was used (200 – 300 lux). Light intensity was measured with a digital luximeter Model-LD-204 (Instrutherm Instrumentos de Medição Ltda., São Paulo, Brazil).

Light cycles and wheel-running activity recording

Animals were placed in individual cages (40 × 35 × 25 cm) with computer-monitored running wheels (30 cm diameter, 10 cm wide, 0.5 cm between bars). Cages were placed in light-tight, ventilated wooden cabinets (180 × 55 × 50 cm) with a timer control for the lighting conditions. Initially, the LD cycle was the same as in the animal facility (light provided by two 30-W fluorescent bulbs, 400 – 500 lux). As explained in more detail below, in Experiment I a skeleton photoperiod (SP) was used whereas, in Experiment II, a typical 12:12h LD cycle was used. Dim light (15 – 25 lux), when necessary, was provided by three incandescent lamps (100 W) connected to a dimmer (Exatron Exata 500 W). When the activity rhythms were stable and synchronized, animals of each condition were randomly assigned to one of two groups. One group was trained/tested during the active phase (2 – 3 hours after activity onset; Active group) and the other group was trained/tested during the inactive phase (14 – 15 hours after activity onset; Inactive group).

Wheel-running activity was continuously recorded with a homemade computer program. Each turn of the wheel activated a micro-switch that was registered as one pulse of activity. The resulting data were analyzed and visualized using ClockLab (Actimetrics, Inc. Evanston, IL, USA). The activity data were displayed in actograms and were used to monitor entrainment to the SP or to the LD cycle, and to determine if the experience in the water maze affected rhythmicity. Cage cleaning (changing the tray underneath) occurred when the animals were in the water maze, to avoid disturbing them at other time points.

Behavioral procedure in the water maze

The task consisted of placing the rat near the side of the pool, facing the wall at one of the starting locations and allowing it to swim until the platform was found or until 120 s had elapsed without finding the platform, whichever occurred first. In the latter case, the animal was manually guided to the platform, where it remained for a 10 – 15 s period. The rat was

then dried thoroughly with a towel and returned to its cage where it remained until the next trial. In the reference memory version of the task, the platform was located in a single, fixed position in the center of the northwest quadrant. The starting locations varied between the cardinal points south, southeast, east, and northeast in random order for each day. In the working memory version, the platform location was changed on each day. Three different starting locations were used in a different order each day. In the first trial on each day of the working memory task, the rats reached the platform by chance and scanning. At the end of this first trial, however, the animals obtained the information about the platform location, and the subsequent trials required matching to the position for that day (Morris & Frey 1997; Xavier et al. 1999). Performance of the animals in the task is not relevant for the purpose of this paper and consequently is reported elsewhere (Valentinuzzi et al. 2004).

Data analysis

Circadian data analysis was done using the ClockLab software. Data for each 24 h cycle were divided into 10 min bins. Determination of daily activity onset was required to determine precision of activity onsets. The software determined the 20th percentile activity level, that is, the activity level that exceeded exactly 20% of all non-zero counts. In other words, the animal was considered active (rated as 1) during each 10-minute interval only when activity exceeded the 20% of the total activity, whereas it was considered inactive (rated as 0) during any 10-minute interval if the activity level was lower than the 20% of the total activity. This strategy eliminated random ''turning of the wheel'' that may occur due to external interference like increased human day activity in the animal facility. The activity record was then converted into a stream of 1's and 0's depending on whether each count exceeded or fell below the 20% percentile. This coded activity record was then fitted with a template that consisted of 10 hours of inactivity (0's) followed by 1 hour of activity (1's) (ClockLab software manual). Data from our laboratory and actograms obtained from the literature (e.g., Groot & Rusak 2000; Devan et al. 2001) reveal that rats typically show poorly consolidated active phases, characterized by high onset variability and many bouts of activity. This is in opposition to other laboratory species like hamsters (e.g., Janik & Mrosovsky 1993; Janik et al. 1994) and mice (e.g., Valentinuzzi et al. 1997, 2001), which show extremely precise onsets. Due to this, this 10 h of 0's and 1 h of 1's template was selected since it was the one that best fitted activity onsets, showing good agreement with visual inspection of the actograms. The software moved the template one point at a time across the entire record calculating at each point the sum of the multiplied points (the ''dot product''). Wherever the template matched the record, a high value was obtained; wherever the template did not match, a low number was obtained. In this way, a graph of the dot product as a function of location of the template was obtained that indicated how good the match was. This graph amounted to a ''cross-correlation'' between the template and record. The red dots (onsets) showed the location of the maximum of the cross-correlation function for each circadian day.

For determination of variability of activity onset, a least-square regression line was fitted to the onsets of the days previous to and during the water maze sessions. The standard deviation of the horizontal distance of the onset points to the fitted line was registered. In other words, precision of activity onset was defined as the daily deviation of the onsets from each animal's fitted regression line. A grand mean of variability was obtained for each data set of each group. The mean daily deviation from each animal's regression line was analyzed with repeated measures ANOVA with grouping variable (active and inactive groups) as the between-subjects factor and Pre-Test/Test (Experiments II) phases as the within-subjects factor. A contrast

analysis following ANOVA revealed differences between groups and test phases (Sigma Stat 1.0, Jandel Scientific).

When necessary, activity rhythm periodicity was calculated by the ClockLab software with a Chi Square periodogram (Zar 1986). For each period value on the *x*-axis of the graph, the data were cut into segments with that period. These segments were averaged to give an activity profile, and the standard deviation of the profile was calculated. The more consistent the waveform in each segment, the larger the signal (standard deviation). This standard deviation was then normalized by the standard deviation of the entire data set. The confidence level (alpha) was 0.01; that is, the chances of random data exceeding the chi-square line at any one point, was 1/100.

Results are reported as means $\pm$ standard errors ($X \pm SE$).

Experiment I

In this experiment, we analyzed the effect of daily trainings sessions in the water maze on the rhythm of locomotor activity of animals submitted to a skeleton photoperiod.

Methods

Twelve rats were placed in individual running wheel cages and left in a LD cycle for 45 days. This long interval was due to reasons other than the purpose of the present experiment. After this interval, the 12 h dark phase was replaced by 12 h of dim (15 – 25 lux) light; this condition continued for two cycles (see Figure 1). Immediately thereafter, a skeleton photoperiod (Pittendrigh 1965), consisting of two 15 min bright light pulses separated by 11.5 and 12 h of dim light (i.e., 0.25 h bright:11.5 h dim:0.25 h bright:12.0 h dim), was established and continued for the duration of the training phase. The purpose of using this type of synchronizing agent was to allow training of the animals at different circadian times, but under identical illumination conditions. After two days in the skeleton photoperiod, training in the reference memory version of the water maze task started. Training consisted of two trials per session (inter-trial interval of 10 minutes). After 15 days of daily training in the water maze, the animals were left in constant dim light in order to establish the phase of the free-running rhythm.

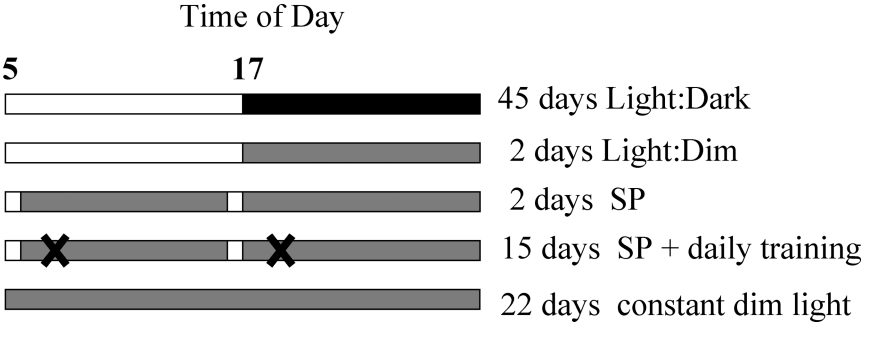

Figure 1. Schematic representation of the different illumination conditions to which animals from Experiment I were submitted. Time is plotted across the horizontal axis (each bar represents 24 h). White areas indicate lights-on, black areas indicate lights-off and gray areas represent dim light. The crosses indicate the times of training for each group. The number of days the animals spent in each condition is indicated on the right. SP: skeleton photoperiod.

Results and discussion

Figure 2 shows wheel-running activity of four rats trained in the reference memory version of the water maze every day during their inactive phase (A), and four rats trained during their

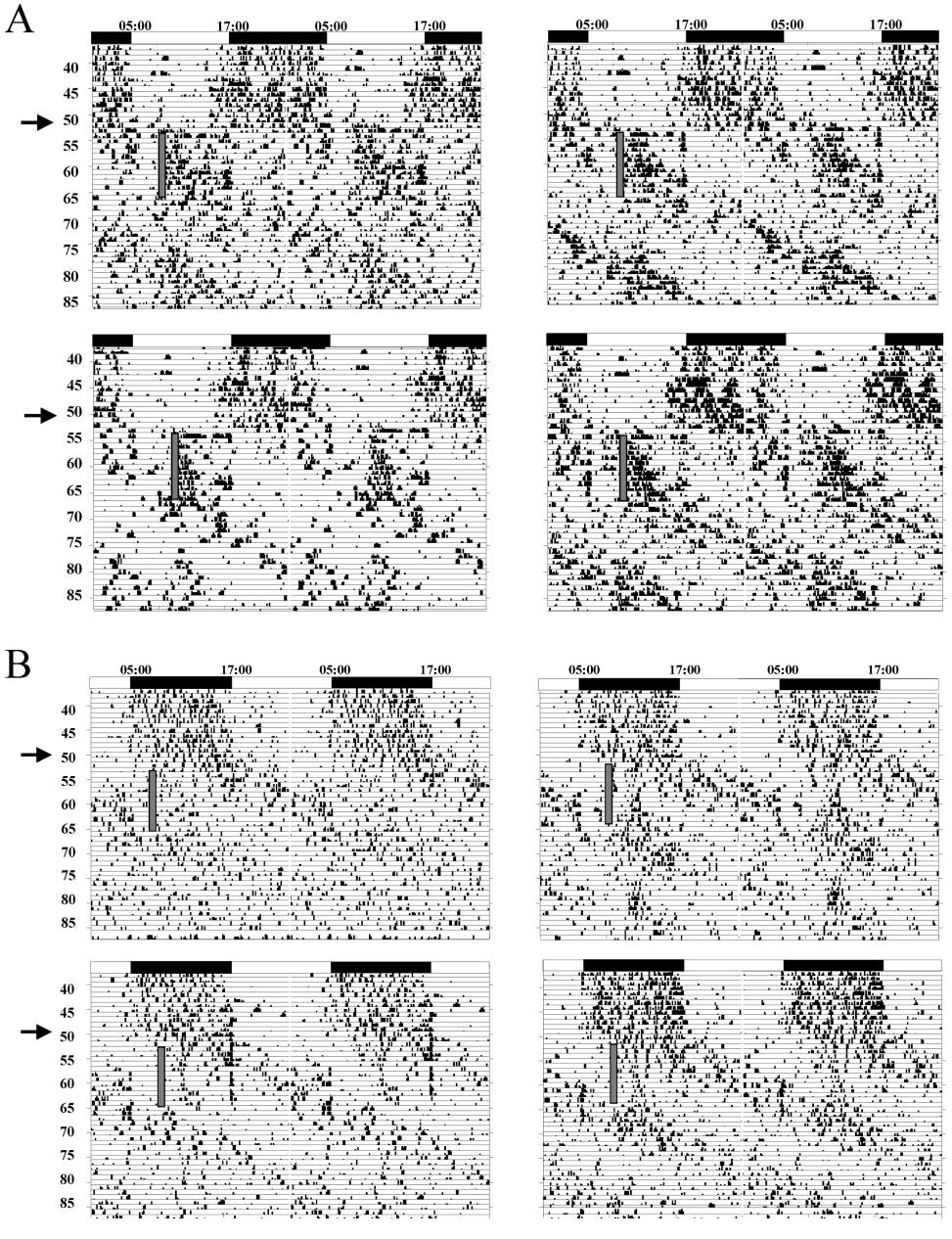

Figure 2. Representative actograms of four animals of Experiment I trained in the reference memory version of the water maze task every day during their inactive phase (A) and four animals trained during their active phase (B). Time is plotted across the horizontal axis (48 h per line), and successive days are plotted beneath one another. The bar on the top indicates the initial light:dark cycle. The arrow indicates when the skeleton photoperiod started. The vertical gray bars indicate when training in the water maze task occurred.

active phase (B). During the first days in a normal LD cycle a typical pattern of wheel-running behavior was observed, where most of the activity was concentrated during the dark phase. Periods during this phase varied from 23.85 h to 24.6 h. Values were not exactly 24 h (as expected in synchronized animals) due to the onset variability that characterizes rats' actograms ($\pm$120 min). The horizontal arrows on the left of Figure 2 indicate when the skeleton photoperiod was initiated and the vertical gray bars indicate when daily training occurred. Immediately after the first session, animals trained in the inactive phase show an apparent abrupt phase shift in activity that was maintained while daily training occurred (Figure 2A). In this group of animals, the daily experience in the maze gave the impression of being a potent *zeitgeber* (controlling phase and period) of the circadian rhythm of locomotor activity (but see below). These animals had no previous experience in the water maze; consequently, the experience in the pool may have represented an extremely stressing/arousing stimulus due to its novelty, leading to an increase in the animal's general activity. It is well known that physical activity can directly affect the biological clock producing significant phase-dependent advances or delays in the circadian rhythm of locomotor activity (Edgar et al. 1991). Period variability during the 15-day training phase was significantly decreased relative to the previous LD cycle/non-training phase; values varied from 23.95 to 24.14 h. In other words, compared to the LD cycle, daily training seemed to control more precisely the overt activity rhythm. If the daily training sessions were truly affecting the clock, phase advancing the active phase, this would mean that the animals that we intended to test during their inactive phase were actually being trained during the active phase. This becomes a relevant issue in experiments that intend to analyze time effect in performance.

Animals trained daily during their active phase had a very mild response to the training sessions (Figure 2B). The apparent phase advance in activity, observed in the inactive group, was not detected in the animals trained during the active phase; instead, short and less intense post-training bouts of locomotor activity were observed. In other words, the novel experience in the water maze seems to have produced an abrupt phase shift of the circadian rhythm of wheel-running activity only of the animals tested during their inactive phase (consistent with the phase-response curve for non-photic stimuli; Mrosovsky et al. 1989). Additionally, in the active group, a free-running component was present, apparently from the time the skeleton photoperiod started. This free running component was confirmed with the periodograms, with values varying between 25.25 h and 26.25 h during the training phase.

After training was completed in both groups, animals were submitted to constant dim light. In these conditions, periodograms revealed a free-running rhythm of 24.24 – 25.05 h (Figure 2). Inspection of the actograms of both groups revealed that what seemed to be a phase shift in the inactive group was probably a direct response of the animals to the experience in the task – that is, a masking effect. Masking is considered to occur when the external agent (in the present case, the experience in the water maze) has a direct effect in the behavior (i.e., locomotor activity) without exerting its effect through the biological clock. By contrast, entrainment implies a clock effect, which controls behavior (Mrosovsky 1999). We believe masking was the cause because the free-running component continued undisturbed during the days in which water maze testing was occurring despite the post-training component. Another observation that favors a masking hypothesis is the immediate "phase advance" after the first training session of the inactive group. A re-entrainment process typically shows a gradual change, expressed as a few days of transients (Boulos et al. 1980); this was not the case for these data. We considered, therefore, that the experience in the water maze masked the activity rhythm and that the phase difference between both groups was a water-maze-induced masking effect. During the inactive phase, masking was much more evident, to the extent that it gave the impression of a radical phase shift. Also, it

maintained a period close to 24 hours, as if daily trainings were acting as a potent *zeitgeber*. On the other hand, during the active phase, water-maze-induced masking resulted in short and less intense post-training bouts of activity. Phase-dependent masking effects are a well-known phenomenon in the literature. For example, this was observed in the response to light pulses of canaries entrained to a LD cycle and in the response to dark pulses of free-running hamsters (e.g., Aschoff 1988); in both cases, the amount of masked activity was clearly dependent on circadian phase. To our knowledge, this phenomenon has never been demonstrated previously in the water maze.

The unexpected result in this experiment was the free-running component despite the presence of the skeleton photoperiod. This is better visualized in the active group due to the less intense masking effect upon the animals tested in this phase. For some unknown reason, the two 15-min bright light pulses (separated by dim light) used here were not strong enough to promote synchronization. We can propose two possible explanations for this. First, it is possible that the genetic background of the group of animals used could have altered their sensitivity to this type of synchronizer. This idea is based on the fact that we have been able to use the same protocol to synchronize animals obtained from another source. Another possibility is related to this zeitgeber's synchronizing strength in this particular species. Data from the literature show that rats successfully entrain to skeleton photoperiods; however, to our knowledge, animals have not been submitted simultaneously to other potential non-photic synchronizers. Again, referring to the same group of animals mentioned above, we have observed an interesting trait. These animals were submitted to the same protocol as that used in Experiment I of the present manuscript, but using a social memory task instead of the water maze task. All the animals that had successfully synchronized to the skeleton photoperiod, free-run as soon as the daily behavioral testing started, despite the fact that the skeleton photoperiod was still present. One interpretation of this could be that the skeleton photoperiod is a weak zeitgeber for this species; it can synchronize individuals, but as soon as another potential zeitgeber is present, the skeleton photoperiod – SCN coupling becomes broken.

The important point from Experiment I is that we tested free-running animals in the water maze and that, in these conditions, a phase-dependent masking effect was observed.

Experiment II

In the previous experiment, rats maintained in running-wheel cages and trained in a reference memory task every day at the same time showed changes in rhythmicity associated with the novel experience in the pool. In the present experiment we investigated if single sessions in the water maze (using the working memory version of the task) could have an effect on the rhythmicity of synchronized animals. Since the skeleton photoperiod used in Experiment I did not seem to retain synchronization, we used a LD cycle in which the dark phase was represented by dim light instead of total darkness. Two different groups were tested. One group had no previous experience in the water maze and consequently the novelty of the experimental situation could be considered an arousing stimulus. The second group underwent a phase of adaptation to the water maze. This manipulation was intended to decrease the novelty of the test situation and, in this way, to determine if familiarity with the task would be sufficient to avoid interference with the overt activity rhythm.

Methods

Group 1. A total of 24 rats were used. Half of the animals were tested during the active phase and the other half during the inactive phase. The Active subgroup was submitted to a 3 hour

phase advance in order to facilitate water-maze testing. Five days later all animals were placed in the running-wheel cages in the LD cycle, as in Experiment I, except that the 12 h dark phase was replaced by 12 h of dim light (15 – 25 lux). In this way, water-maze testing occurred in an environment with the same amount of light relative to the light-cycle chamber, so avoiding abrupt changes in the lighting conditions during testing. Working-memory testing started seven days later and lasted three sessions, one session per day, with an inter-session interval of three days; each session consisted of three trials per day (the inter-trial interval was 30 minutes).

Group 2. Twenty-four rats were submitted to a phase of adaptation to the Morris water maze. This consisted of training with the reference memory version of the task over the course of seven days, with four trials per day (the inter-trial interval was 10 minutes). Four trials per day instead of two were used in order to increase exposure of the animals to the experimental situation. Our intention with this was to facilitate habituation, if this applies to such a task. Half of these animals, corresponding to the Active group, were submitted to a three hour phase advance as occurred in Group I. Five days later, all animals were placed in the individual running-wheel cages in the same LD conditions as for Group 1. Working memory testing occurred nine days later and consisted of three trials per day (the inter-trial interval was 30 min). It was run over the course of five days with a 1 – 2 day inter-session interval.

Since direct observation of the actograms revealed an increase in activity onset variability as soon as testing in the water maze started, this was the parameter used for analysis. Regression lines were drawn through the onsets of two different periods of the actograms; a 6 – 8-day period before the beginning of the tests in the water maze (Pre-Test phase), and a 10-day period when testing was taking place (Test phase).

Results and discussion

Figure 3 shows four representative actograms of rats from Group I (animals with no previous experience in the task) tested during their inactive phase (A) and four rats tested during their active phase (B). The six-day Pre-Testing phase was taken as a baseline representative of undisturbed animals. The baseline Pre-Test phase was characterized by precise activity onsets. As soon as testing in the maze started, onset variability increased significantly. As can be seen in the actograms, the daily deviations of onsets from the fitted lines increased during the Test phase. This increased onset variability seemed to be greater in animals tested during their inactive phase; however this effect did not reach significance. Figure 4 shows the onset variability of the Pre-Test and Test phases of both Inactive and Active groups. The ANOVA revealed a significant Test effect ($F_{1,22} = 20.79$; $p < 0.0002$) on onset variability, but no significant interaction.

Figure 5 shows representative actograms of wheel-running activity of four rats of Group 2 (animals with previous experience in the pool) tested in the working memory version of the water maze during their inactive phase (A) and of four rats tested during their active phase (B). Results were generally identical to those observed in Group 1. That is, as soon as testing started, onset variability increased. Again, this effect seemed to be greater in the animals tested during their inactive phase. These observations are confirmed in Figure 6 where the mean values of onset variability during the Pre-Test and Test phases in both inactive and active groups are shown. The ANOVA revealed a significant Pre-Test/Test effect ($F_{1,19} = 22.4$; $p = 0.0002$) and a trend for an interaction effect between phase and time of testing ($F_{1,19} = 3.4$; $p = 0.0786$).

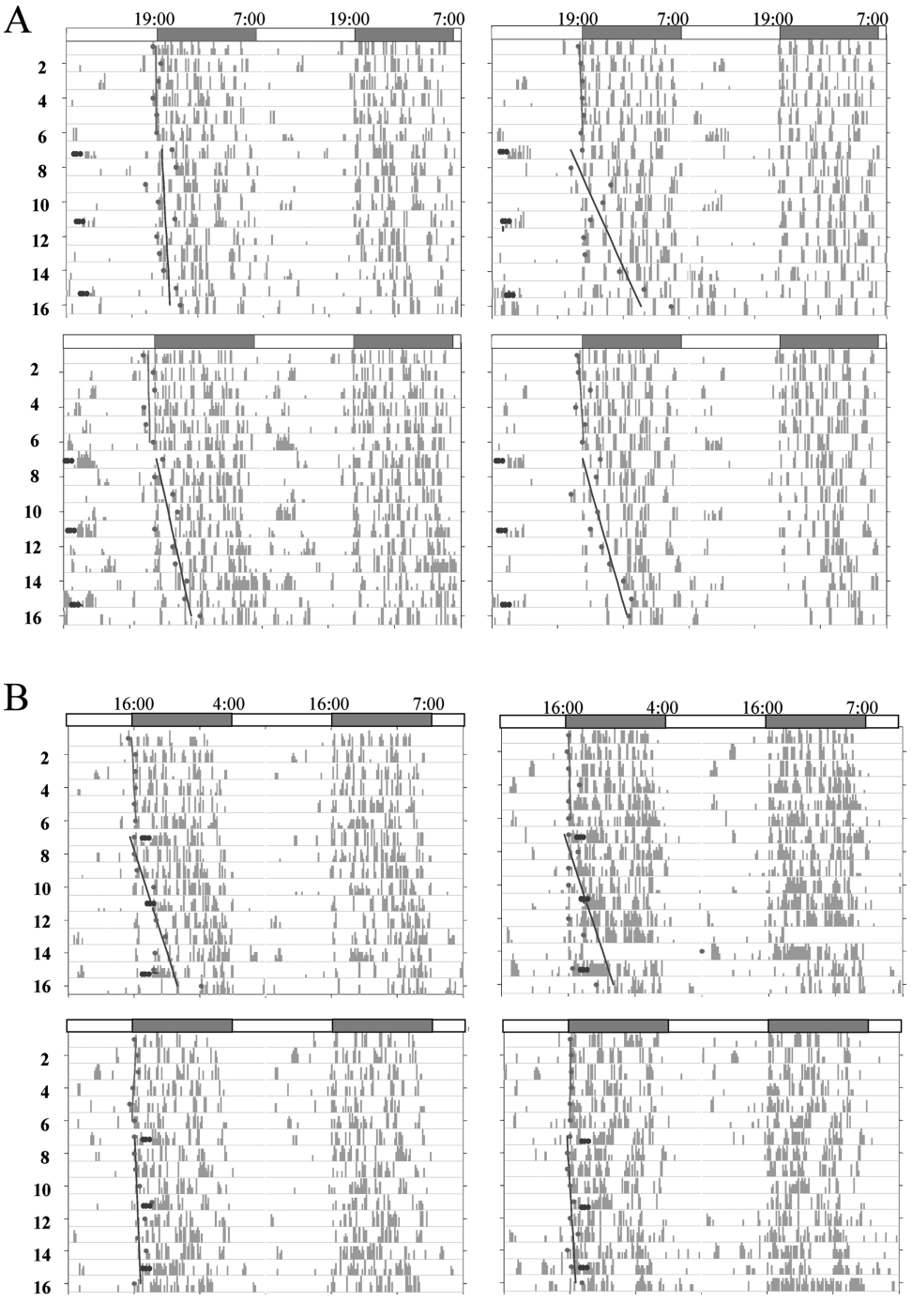

Figure 3. Representative actograms of four animals of Experiment II, with no previous experience, tested for working memory in the water maze during their inactive phase (A) and four animals tested during their active phase (B). Time is plotted across the horizontal axis (48 h per line), and successive days are plotted beneath one another. The bar on the top indicates the intense-light:dim-light cycle. The blue dots represent testing trials of each session. The red dots represent activity onsets. The red lines are the regression on the onsets during the 6-day Pre-Test phase. The blue lines are the regression on the onsets of the 10-day Test phase.

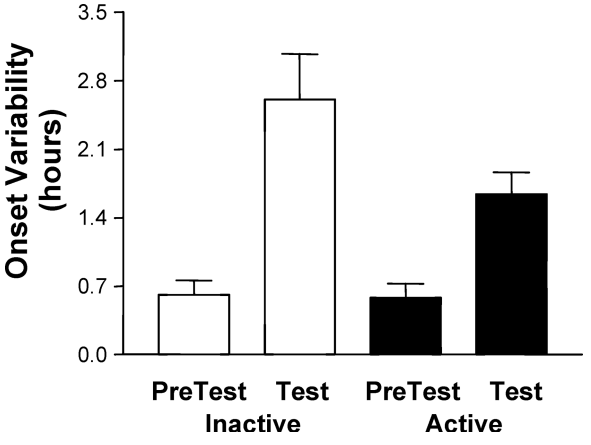

Figure 4. Onset variability expressed in hours during the 6-day Pre-Test phase and during the 10-day Test phase of animals of Experiment II, with no previous experience, tested during their inactive phase and those tested during their active phase.

Experience in the water maze affected the circadian rhythm of locomotor activity; significant alterations in the precision and stability of rhythmicity occurred. This suggests that the experience in the water maze may be a behaviorally-arousing stimulus that can alter the expression of rhythmicity. This non-photic effect occurs despite the presence of a strong *zeitgeber,* such as the LD cycle, reinforcing the strong arousal-inducing characteristics of this task. Additionally, the experience in the water maze produced in animals of group I a direct effect, which was expressed as running in the wheels immediately after testing. It might be that this activity induced by the water-maze affected the cycles that followed.

The literature on non-photic synchronization has discussed extensively possible non-photic stimuli that could affect the circadian system. The main factor has been considered to be the internal activation or arousal that the stimulus causes in the animals, and its resulting increase in physical activity. We are not saying that the experience in the water maze is directly responsible for ''synchronizing'' the animals; however, we do suggest that the activation or arousal that the experience in this task produces in the animals can lead to an increase in activity, and this can affect the expression of the normal endogenous circadian rhythm of activity. In other words, submitting the animals to such an arousing situation acts either through the pacemaker or through overt behavior. The increase in onset variability might have two explanations. First, it may indicate a conflict of stimuli between the LD cycle and an unexpected, alien situation. Alternatively, it could be the result of a decrease in the coupling force between the LD cycle and internal oscillators.

In Group 2, the experience in the water maze also significantly affected the circadian rhythm of locomotor activity, despite the fact that the animals had become familiar with the task situation. The novelty of the situation may not be the cause of circadian changes or the adaptation phase used here was not sufficient to familiarize the animals with the task. However, these animals did not show post-training bouts of activity. This suggests that the adaptation phase led to a degree of familiarity with the experience, as a result of which the level of arousal produced in the animals was lower. Also, the change in onset variability elicited by the experience in the maze may not be caused by the post-training bouts of activity.

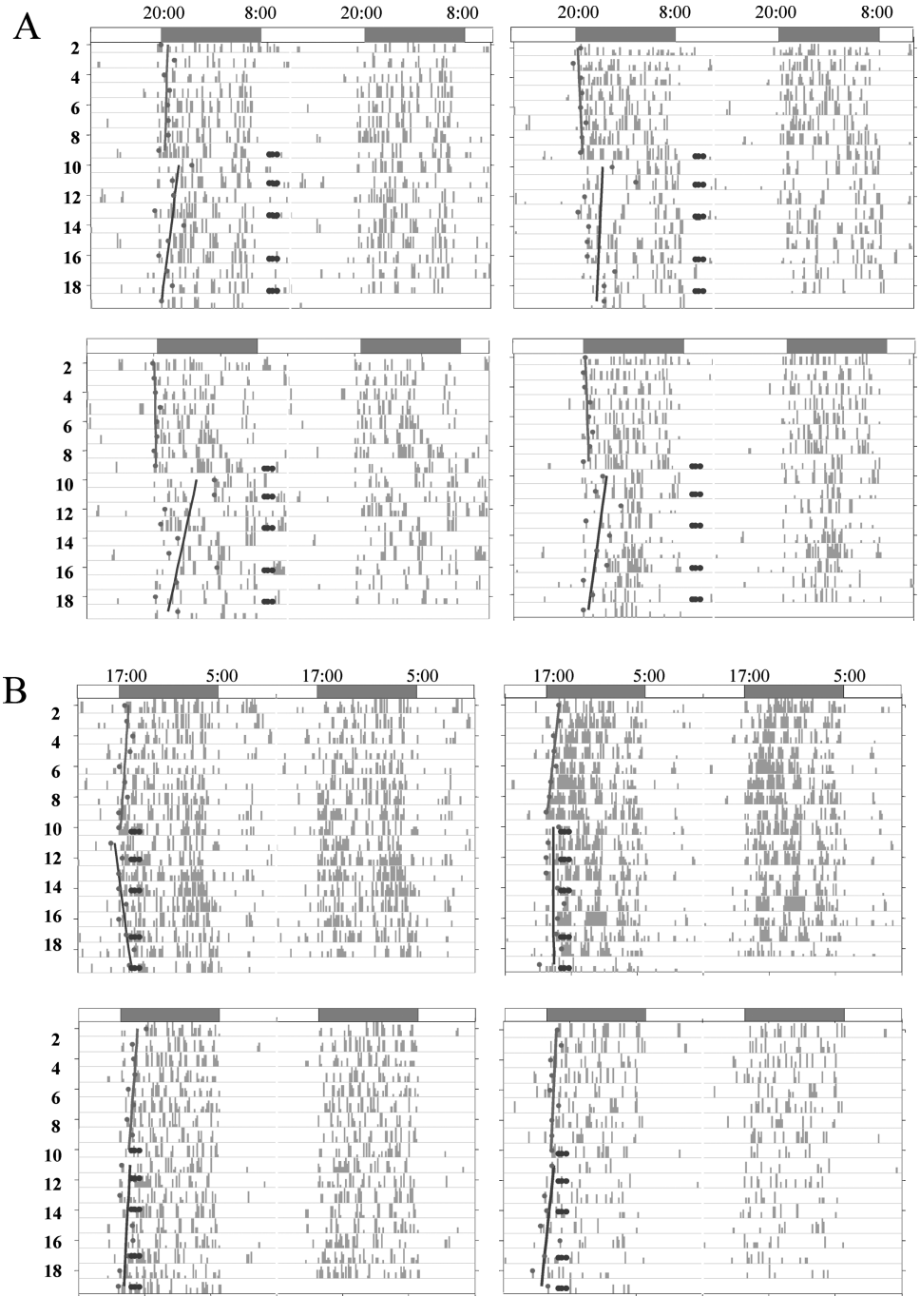

Figure 5. Representative actograms of four animals of Experiment II, with previous experience, tested for working memory in the water maze during their inactive phase (A) and four animals tested during their active phase (B). Time is plotted across the horizontal axis (48 h per line), and successive days are plotted beneath one another. The bar on the top indicates the intense-light:dim-light cycle. The blue dots represent the trials of each session. The red dots represent activity onsets of each cycle. The red lines are the regression on the onsets during the 9-day Pre-Test phase. The blue lines are the regression on the onsets of the 9-day Test phase.

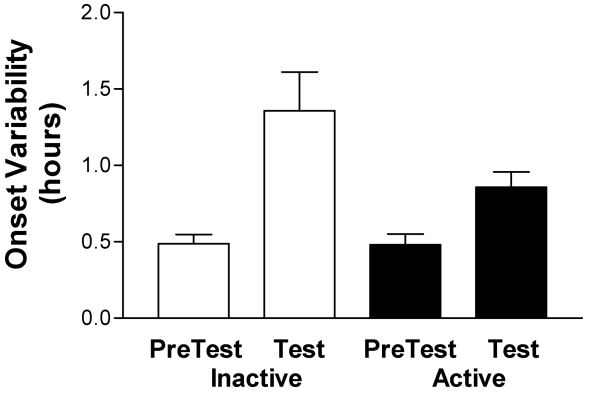

Figure 6. Onset variability expressed in hours during the 8-day Pre-Test phase and during the 10-day Test phase of animals of Experiment II, with previous experience, tested during their inactive phase and those tested during their active phase.

General discussion

Data from these two experiments show that the experience in such an arousing-inducing situation as the water-maze task can lead to significant changes in the expression of the circadian rhythm of locomotor activity. This effect becomes very obvious if the experience in the task occurs every day at the same time and if the animals are free-running, as was observed in Experiment I. Circadian changes can occur despite the presence of a potent synchronizer such as the LD cycle. In other words, when testing animals on behavioral tasks, especially in those that have a stress-generating effect or in tasks that imply certain levels of forced physical activity (as is the case with the water maze), care should be taken with respect to possible changes in rhythmicity.

Non-photic experiences are capable of changing the phase of circadian clocks (Mrosovsky 1996; Turek 1989; Mrosovsky et al. 1989). The main focus of these studies has been on stimuli that generate physical activity *per se* or some associated variable that affects the clock. There are numerous ways of making animals active, including sexual arousal, opportunities to hoard, novel running-wheels, drugs that stimulate activity, dark pulses in nocturnal animals, social interactions between males, and cage-changing. There have been proposals for the existence of a non-specific non-photic system that can be activated in a variety of arousing situations (Mrosovsky 1996; Turek 1989; Mrosovsky et al. 1989).

The observation of the present study, that the most obvious effect on rhythmicity was observed in the animals tested during their inactive phase, makes sense when the phase response curve of activity-inducing stimuli is considered (Mrosovsky et al. 1989). That is, a behaviorally-arousing event occurring at one phase of the circadian cycle affects the pacemaker differently from the same event occurring at another phase of the cycle. As mentioned before, non-photic phase response curves have not been determined for rats. However, the sensitivity of the circadian system to this kind of stimulus in hamsters is mainly during the inactive phase (Mrosovsky et al. 1989).

Non-photic stimulation may affect the circadian system in several ways. For instance, considering the water-maze test, performing this on an animal during its inactive phase involves interrupting its rest, picking it up and exposing it to novel surroundings. Strong phase shifts have been reported when a particular situation is completely novel, for example, exposure to a wheel in animals that normally do not have access to one

(Turek 1989). However, in our Group 2 of Experiment II, the novelty of the task situation was probably not the cause of the behavioral activation, since these animals had previously been submitted to an adaptation phase to the maze and the whole manipulation/context involved with the task.

The activity generated by the water-maze task could be involved in phase shifts. Correlations have been shown involving the amount/duration of the activity and the degree of phase shift (Mistlberger 1991). However, in the present case, activity seems unlikely to have a significant effect since the time the animals spent swimming was very short – from a few seconds of forced swimming (in most cases) reaching a maximum (in very few cases) of six minutes per day (three trials of two minutes each). Activity-induced phase shifts are typically produced by long periods of activity, for example, 1–3 hours of novelty-induced wheel running (Mrosovsky 1996). However, not only the duration but also the intensity of activity may affect the system. When comparing animals confined for three hours in a running-wheel, phase shifts were more pronounced in animals that ran more (Turek 1989). In Experiment II, animals tested during the inactive phase swam significantly faster than those tested during their active phase (see Valentinuzzi et al. 2004). In other words, the more intense swimming of the animals tested during their inactive phase could be the cause of the more pronounced changes in rhythmicity.

Another variable potentially involved in the phase shifts is the motivation and arousal generated by the water-maze test. Several authors propose that arousal may be the critical factor in the behavioral effect of pacemakers (e.g., Mrosovsky 1996). However, sometimes it is not sufficient to induce phase shifts. For instance, Mistlberger (1991) observed only marginal effects of periodic forced activity as a synchronizer, while Beersma et al. (1987) saw no signs of entrainment in rats forced to walk each day for 3 h by slow rotation of their cages. Mistlberger (1991) suggested that the motivational significance may be important for affecting the clock. Some have suggested that the arousal has to be of a rewarding kind, leading to spontaneous activity. Bolles et al. (1974) were unable to condition rats to anticipate and avoid shocks occurring once a day at a fixed time, but had no difficulty in teaching the animals to press a bar before daily feeding. On the other hand, birds can be entrained by daily rattling of the bars of their cages, which is likely to be an alarming experience for them (Reebs, 1989). Whether rats find the water maze rewarding or alarming remains unclear; however, it does seem to produce a strong arousal that leads them to intense running in their wheels.

Even though body temperature increases with arousal and with activity, it does not appear to be sufficient to produce behavioral shifts (Herzog & Huckfeldt 2003). In the case of the water maze, animals were placed in water at $26 \pm 1^{\circ}$C. It could be that the increased wheel-running activity after swimming in the pool has a thermoregulatory component especially in the animals tested during their inactive phase, a circadian phase characterized by a lower body temperature. However, other studies have suggested that running for thermoregulatory purpose does not seem to have a motivational component strong enough to produce circadian shifts (Janik & Mrosovsky 1993).

In summary, the running wheel activity in the present group of experiments suggests that the experience in the water maze task can lead to changes in the expression of rhythmicity. The experience in the water maze apparently alters the motivational state of the animals. Animals with no previous experience, and tested either during the active or inactive phases, show immediate bouts of activity in their wheels after maze testing. The fact that these running bouts are voluntary strongly suggests a true motivational change. Most importantly, this is true despite the presence of a potent synchronizer such as the LD cycle. In other words, when testing animals in certain tasks, especially in those that have a stress-generating effect or in tasks that imply certain levels of forced physical activity, care should be taken with respect to possible changes in rhythmicity that might be produced.

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