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Temporal Control of Spermatogenesis Is Independent of the Central Circadian Pacemaker in Djungarian Hamsters (*Phodopus sungorus*)[1]

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ABSTRACT

In mammals, the duration of the cycle of the seminiferous epithelium (DCSE) largely differs between species, but is remarkably stable within a species, usually showing variations of 1%–3%. It is difficult to change the DCSE, e.g., by hormones or chemicals. Initial experiments, employing quantitative RT-PCR, aimed at investigating the diurnal profiles of the clock genes *Arntl* (previously called *Bmal1*) and *Per1* in testes and kidneys of Djungarian hamsters (*Phodopus sungorus*). While the testicular levels of *Arntl* were almost constant, clear diurnal variations were identified for *Per1*. In order to clarify whether day length (T-cycle) is a factor for DSCE, adult male hamsters (n = 20 per group) were exposed to normal (T = 24 h), prolonged (T = 25 h), or shortened (T = 23 h) T-cycles, with cycles thus being longer or shorter by 4.2% compared to the normal condition. Exposure lasted for 43 days, during which the activity of the animals was recorded to confirm entrainment. DCSE was estimated by incorporation of bromodeoxyuridine in dividing cells and the immunohistochemical localization of labeled cells in stages I–XII of the seminiferous epithelium. Despite the low variability of the results and the close agreement with previously published data, no effects of prolonged or shortened T-cycles on DCSE could be identified (24 h: 7.98 ± 0.05 days; 23 h: 7.94 ± 0.04 days; 25 h: 7.91 ± 0.03 days; $P > 0.05$). The results strongly indicate that the high temporal precision of spermatogenesis is independent of the central circadian clock.

circadian rhythm, sperm maturation, spermatogenesis, testis

INTRODUCTION

The production of sperm cells is a fascinating process during which spermatogonial cells undergo different mitotic and meiotic divisions and further changes during spermiogenesis, after which the elongated spermatids are released. It has long been known that this process is highly organized in space and time [1]. The cellular associations of spermatogenesis have been described in a variety of rodent species, e.g., the rat, the Djungarian hamster, and the golden hamster. The duration of the cycle of the seminiferous epithelium (DCSE) differs greatly among species but is remarkably constant within a species. In rodents, the duration of the epithelial cycle has been found to vary between 6.7 days in the bank vole [2] and 17.0 days in the Chinese hamster [3]. Within a species, the variation coefficient of the duration of epithelial cycle is, however, extremely small, e.g., between 0.5% in Djungarian hamsters [4] and 1.6% in Sprague-Dawley rats [5]. These coefficients of variation are all the more remarkable considering that all possible sources of variation, e.g., methodological differences, are already included.

Though not yet entirely understood, the stable temporal pattern of spermatogenesis is probably due to the highly constant duration of the successive steps of germ cell proliferation and differentiation [6].

It is remarkably difficult to change the DSCE experimentally, e.g., by treatment with gonadotropin-releasing hormone antagonists in rats and monkeys [7] or by hypophysectomy in rats [8]. Likewise, hemicastration has no influence [5, 9]. Treatment with chemicals (e.g., 2,5 hexanedione [1% in drinking water for 1 mo] [10]) has been shown to prolong the DSCE by 8.6% in rats. This treatment, however, has severe systemic effects (i.e., a decrease in body weight by approximately 25%), so the effects of the chemical on the DSCE must be interpreted with caution. The only physiological process by which the DSCE is altered can be observed during puberty. It was conclusively shown that the DSCE is accelerated in immature hamsters and rats [11].

The current opinion is that the DSCE is genetically determined because transplanted rat spermatogonia in mouse testes differentiated with cell cycle timing characteristic of the rat [12]. The same principle was shown later in porcine and ovine testis xenografts [13]. A large number of genes are transcribed by testicular cells (for review see [14]), including genes involved in circadian clocks, which have been identified to be expressed with some diurnal differences [15]. A study using transgenic rats has shown that the expression of the clock gene *Per2* in isolated testicular interstitial cells closely follows a 24-h pattern, thus suggesting that those cells are part of a peripheral clockwork [16].

The central pacemaker of mammals is the suprachiasmatic nucleus (SCN) in the brain. It is synchronized with the environmental day length (24 h) by the light/dark cycle via the retinal perception of light. Recent studies have demonstrated that the light for synchronization is perceived by special neurons within the retina containing melanopsin as the chromophore and that the retinohypothalamic tract projects directly into the SCN [17]. Within certain limits (entrainment range), the circadian system can be synchronized to day lengths (T-cycles) different from 24 h [18]. To our knowledge, the Djungarian hamster has not been tested for the entrainment to light:dark cycle periods different from 24 h (T-cycles).

It has long been known that besides this central clock, peripheral clocks exist in almost all organs, including blood cells (for review see [19]). Although peripheral clocks are generally in a stable phase relationship with the SCN pacemaker, they can be dissociated from it by external cues such as food through temporal food restriction [20]. The aim of our investigation was to find out whether the DCSE is influenced by the central circadian clock. An initial experiment

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was performed in hamsters to investigate the expression of two genes (*Per1* and *Arntl*) that represent core clock genes of the central pacemaker in the SCN and that were found to be expressed in a diurnal fashion in testes of Syrian hamsters [21].To test the hypothesis, we have exposed Djungarian hamsters to T-cycles different from the normal 24 h by ±1 h. This difference (4.2%) is considerably larger than the coefficient of variation of the DCSE, so that a possible entrainment would be detectable.

MATERIALS AND METHODS

Animals

Djungarian hamsters (*Phodopus sungorus*; age between 5 and 8 mo at the start of the experiment) used in these experiments were born and raised in our laboratory under a long-day photoperiod (16L:8D). Food (hamster diet No. 7024; Altromin GmbH & Co. KG) and tap water were available ad libitum. For the experiments the hamsters were transferred into a separate room and kept individually in polycarbonate cages on softwood bedding and a handful of hay. Room temperature was maintained at 20 ± 2°C. The Bremen state commission for animal welfare approved the experiments according to §8a of the German animal welfare law (522-27-11/02–00 [90]).

Diurnal Expression Patterns of the Clock Genes Per1 and Arntl

In an initial experiment, hamsters, kept under normal photoperiod (16L:8D), were killed after deep CO_2 anesthesia every 3 h over 24 h (n = 4 per time point). Kidneys and testes were frozen in liquid nitrogen. Ten to 20 mg of each sample was weighted and transferred immediately in buffer RLT (Qiagen). Afterwards the tissues were disrupted and homogenized with an Ultra-Turrax T8 (IKA Werke GmbH & Co. KG) for 20–40 sec. For total RNA extraction, the RNeasy Mini Kit (Qiagen) was used following the manufacturer's protocol for animal tissues. The samples were always kept on ice, and after purification they were stored at −20°C. To remove contaminating DNA, the RNA extracts were treated with the TURBO DNA-free kit (Ambion Inc.) according to the instructions provided in the manufacturer's handbook.

For a quantitative detection of the clock genes *Per1* and *Arntl* in testes and kidneys, specifically designed primers (Eurofins MWG Operon) and the QuantiTect SYBR Green RT-PCT Kit (Qiagen) were used. The following primers were used: for *Per1*, TGGTAGCATCGACTCCTCAG (forward) and GGCACCTGGTAAGTCATCTG (reverse); for *Arntl*, TGGAGGGACTCCA GACATTC (forward) and CTGGATCCTTGGTCGTTGTC (reverse). The total reaction volume of 25 µl of each sample was composed of 0.25 µl QuantiTect RT Mix, 12.5 µl SYBR Green I RT-PCR Master Mix, 0.13 µl (pmol/µl) of each primer, 7 µl RNase-free water, and 5 µl of the diluted sample. During preparation, samples and substances were always kept on ice. All quantitative RT-PCR reactions were performed in the Mx3000P thermocycler (Stratagene). The first step of RT-PCR, the reverse transcription of mRNA into cDNA, took place at 50°C for 30 min. The reverse transcription step was followed by 15 min at 95°C, the initial denaturation step, where the reverse-transcription enzymes were inactivated and the HotStarTaq DNA polymerase (Qiagen) was activated. Subsequently, 40 cycles of denaturation (95°C for 15 sec), annealing (55°C for 30 sec), and elongation (72°C for 30 sec) of the cDNA followed. During elongation, increasing amounts of fluorescent dye SYBR Green I bound to the nascent double-stranded DNA molecules, emitting a fluorescent signal on binding. At the end of the elongation step of every PCR cycle, a fluorescence measurement was performed to monitor the increasing amount of amplified DNA. The excitation and emission maxima were at 494 nm and 521 nm, respectively.

Altered T-Cycles

Sixty adult male Djungarian hamsters were divided into 3 groups with 20 hamsters each. While one group was left at normal T-cycle (group 24 h, photoperiod 16L:8D), one was exposed to a 1-h-shortened (group 23 h; 16 L:7D) and the third to a 1-h-extended T-cycle (group 25 h; 16L:9D) for 43 days. The duration of 43 days for exposure was chosen to ensure that a complete development of spermatogenesis from spermatogonia to elongated spermatids took place. Because the DCSE in Djungarian hamsters has been shown to be 7.9 days [4], and 4–4.5 cycles are needed for the completion of spermatogenesis [22], the duration of the exposure to different T-cycles was sufficient in this respect. Light was controlled by a digital time switch (TR 641 S DCF KNX; Theben AG). The complete time program for the 43 days of each experiment was written with the aid of the PC programming kit Obelisk 2.1 (Theben AG). All photoperiods were of the long-day type (i.e., 16 h of light per day), so that gonadal regression (as a consequence of reduced numbers of light hours per day) was prevented. Exposures were done consecutively in the same room. The hamsters were caged individually with wooden panels between the cages to avoid visual interactions between the hamsters. Passive infrared sensors above each cage recorded the locomotor activity during the experiments to detect if the hamsters were entrained.

Estimation of the DCSE

We followed the procedures described previously [4, 5], with modifications. On Day 35 of exposure, all animals of each group received an intraperitoneal injection of bromodeoxyuridine (BrdU; Sigma-Aldrich Chemie GmbH) at a dose of 250 mg/kg body weight in 0.4 ml saline after brief CO_2 anesthesia. All injected animals recovered within less than a minute without signs of discomfort. Three hours after injection, 10 animals per group were killed by CO_2 asphyxiation. The remaining hamsters were kept in the cages for 8 more days and were killed 8 days 3 h after BrdU injection. By using a time interval corresponding to the reported cycle duration of the Djungarian hamster [4], the most advanced BrdU-labeled cells were expected to be in the same stages of spermatogenesis, compared to 3 h after injection.

After routine histological preparation (fixation in Bouin solution, dehydration, embedding in paraffin), sections (4 µm) of at least three different regions of each testis were prepared and transferred to glass slides (SuperFrost Plus; Menzel GmbH & Co. KG). After the slides were deparaffinized and rehydrated, excess water was removed, and the cross sections were encircled with a liquid pap pen (Daido Sangyo Co., Ltd.) to minimize the needed staining solution volumes. All staining steps were performed at room temperature. Prior to adding primary antibody, cross sections were incubated for 10 min in H_2O_2 to avoid unspecific background staining, washed with tap water and demineralized water, followed by proteolytic digestion with proteinase K (Sigma) for 5 min at room temperature and a Tris buffer at pH 7.4 for 3 min, three times. Subsequently, the primary antibody (mouse anti-BrdU [1:200 dilution; clone Bu20a, code No. MO744, DAKO GmbH]) was applied to the cross sections until they were completely covered (20–30 µl), and the cross sections were incubated for 30 min. Afterwards, they were immersed in buffer for 3 min three times. For further treatment, the ZytoChem-Plus HRP Polymer-Kit (Zytomed Systems GmbH) was used according to the manufacturer's instructions. Subsequent to the immunohistochemical staining, the periodic acid-Schiff (PAS) staining was performed. Cross sections were treated for 15 min with 1% periodic acid (Merck), washed with tap water and aqua dem., and incubated in Schiff reagent (Merck) for 30 min. This technique stains the acrosome of spermatids deep purple and is therefore indispensable for spermatogenic stage identification. Finally, the specimens were counterstained for 2 min with undiluted Mayer hemalaun solution (AppliChem GmbH) and embedded with Mowiol (polyvinyl alcohol; Fluka) that was composed of 100 mM Tris base, pH 8.0, and glycerol (99%). Specimens were covered with 24 × 50-mm coverslips. Negative (with aqua dem. instead of primary antibody-incubated specimen) and positive (specimen with proven BrdU labels) controls were included daily in the staining runs to guarantee the quality and the specificity of the staining method.

According to the criteria established by van Haaster and de Rooij [4], 400 tubular cross sections (of different testicular regions) per animal were analyzed and the stage and BrdU staining frequencies were determined. As described in detail elsewhere [4, 5], the stage frequency and the staining frequencies were used to calculate the DCSE for each animal that was killed 8 days 3 h after injection. The stage and staining frequencies of all animals that were killed 3 h after injection served as reference. All microscopic analyses were done in a blinded fashion, i.e., the slides were coded prior to analyses and decoded only after all data were evaluated.

Statistical Analyses

Percentages of stage and staining frequencies were arcsin transformed to obtain normally distributed data. The transformed data and the raw data for DCSE were analyzed by ANOVA (two-sided) followed by a post hoc Tukey test. All statistical comparisons were performed with the software SPSS (version 17.0; SPSS Inc.). Results are expressed as means ± SEM unless otherwise specified.

RESULTS

Diurnal Expression Profiles of Per1 and Arntl

The expression profiles of the clock genes *Per1* and *Arntl* are shown in Figure 1. In kidneys, both genes are expressed

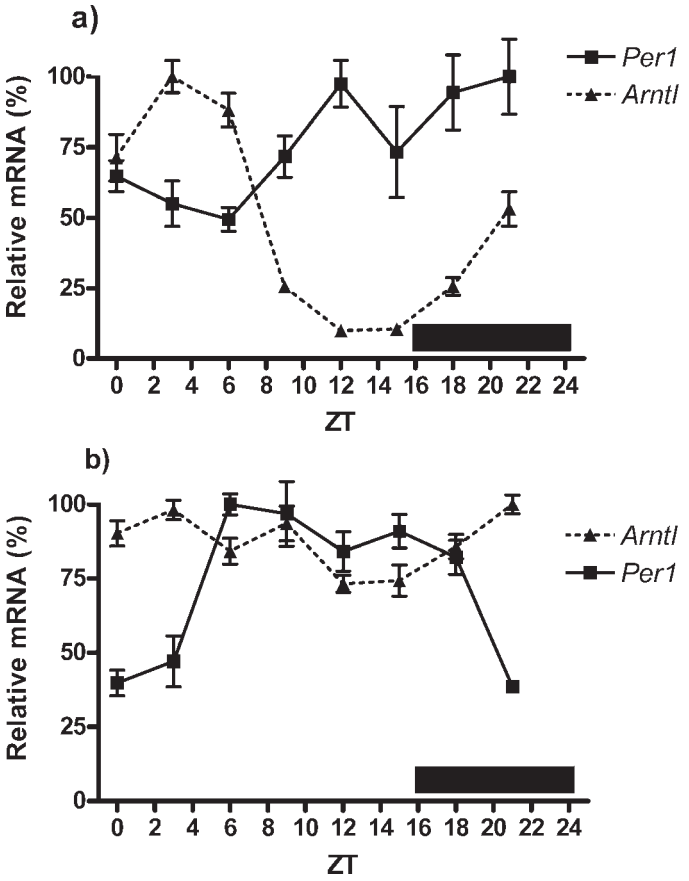

FIG. 1. Diurnal expression profiles of the clock genes *Per1* and *Arntl* in kidneys **(a)** and testes **(b)** of Djungarian hamsters kept at 16L:8D. Shown are the relative data with 100% being the maximum (mean ± SEM, n = 4). The x-axis represents zeitgeber time (ZT). By definition, the time of lights-on is ZT 0 (0300 h). Black bars indicate the dark phase from 1900 h to 0300 h.

with strong diurnal variations and in opposite phases. *Per1* shows maximum expression at zeitgeber time (ZT) 21 (1200 h; 5 h after onset of darkness) and a nadir at ZT 6 (0900 h; 6 h after onset of light), whereas the maximum expression of *Arntl* shows the highest values at ZT 3 (0600 h; 3 h after onset of light) and the lowest values at ZT 12 (1500 h; 12 h after onset of light). In the testes, only *Per1* exhibits strong diurnal variations, with a peak at ZT 6 (0900 h; 6 h after onset of light) and a nadir at ZT 21 (1200 h).

Entrainment to the Altered T-Cycles

Most animals of group 1 (24 h) were entrained, as expected. The only exception was one animal that showed an arrhythmic behavior (data not shown). In group 2 (23 h) 15 out of 20 animals followed the shortened T-cycle. Five animals initially were entrained but started to free run between Days 6 and 16. In group 3 (25 h), only six animals were entrained throughout the experimental time; the others started to free run between Days 7 and 16. Examples of locomotor activity patterns of entrained animals are shown in Figure 2.

Stage and Staining Frequencies, DCSE

The stage frequencies of the seminiferous epithelium are shown in Figure 3 for the three groups. As expected, there were marked differences in the frequencies, with stage VII being most abundant, whereas stages II and III showed the lowest

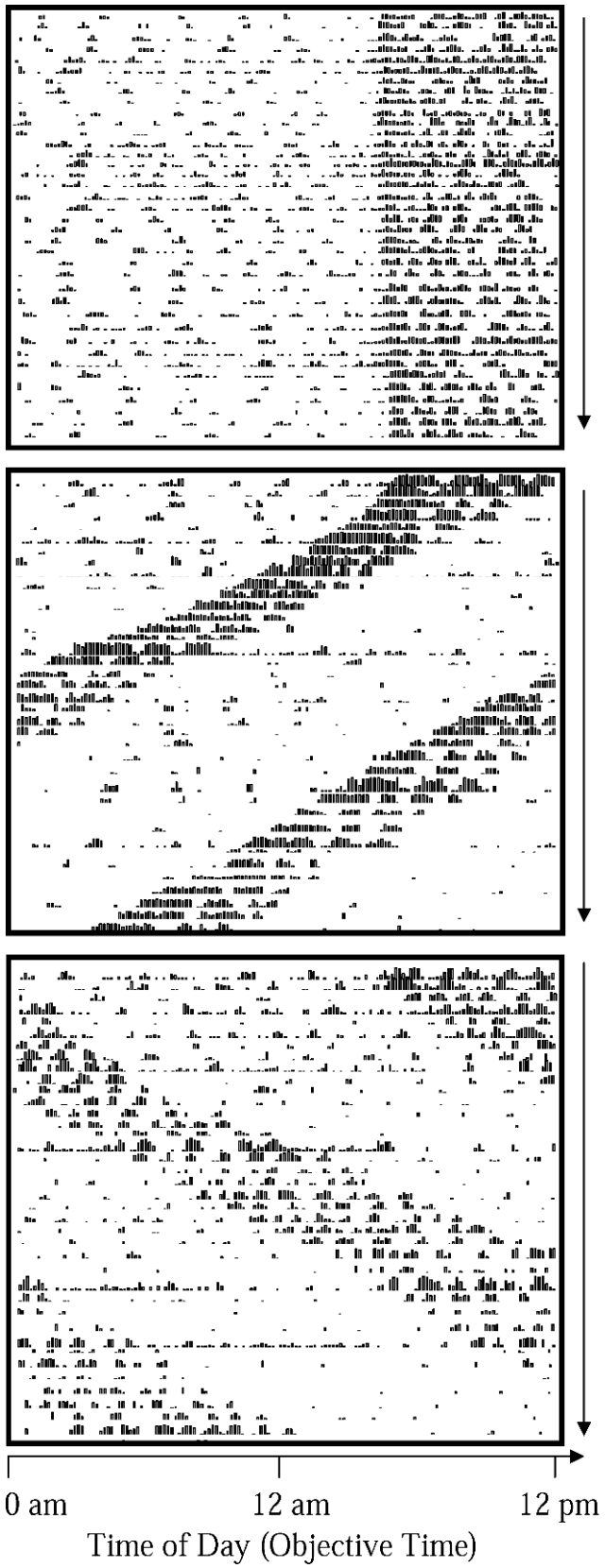

FIG. 2. Examples of actograms of animals that were entrained to the different T-cycles. Top: 24 h (16L:8D); middle: 23 h (16L:7D); bottom: 25 h (16L:9D). Each bar represents the recorded activity within a 10-min interval. For easy visualization, the dark periods are not shown.

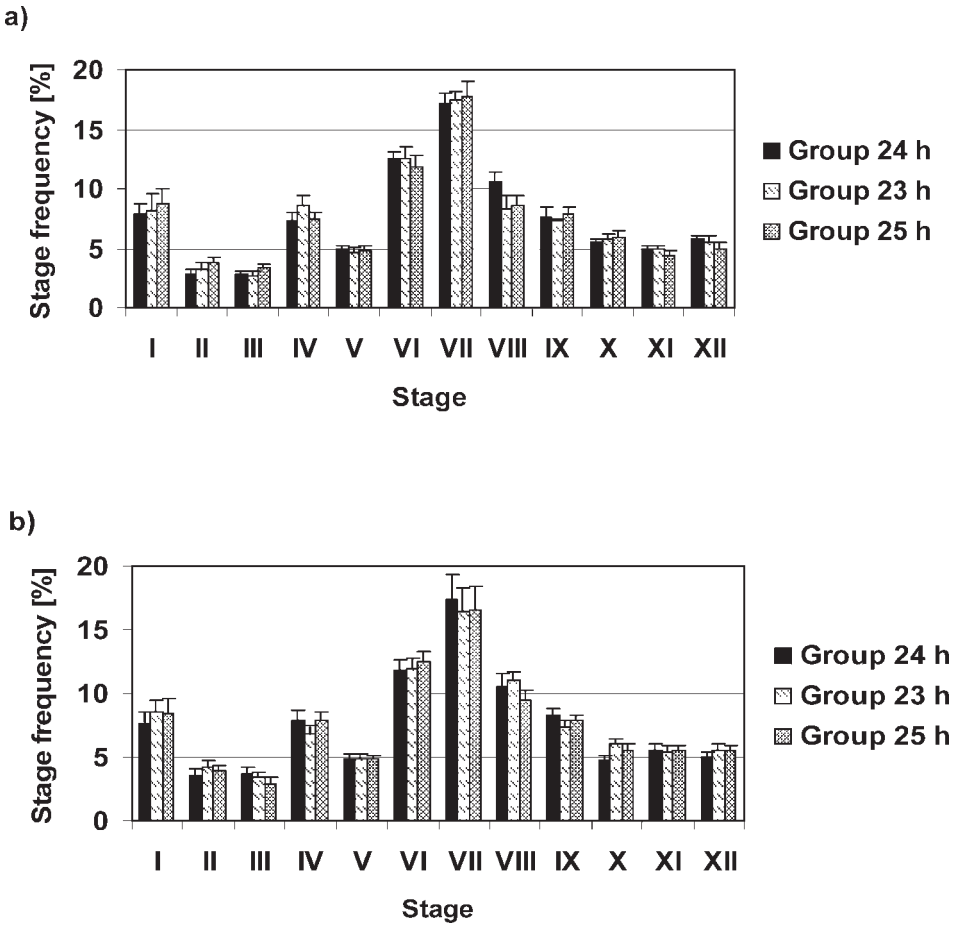

FIG. 3. Stage frequencies of the seminiferous epithelium estimated 3 h **(a)** and 8 days 3 h **(b)** after injection with BrdU. Between the groups, no significant differences were identified.

frequencies among the 12 stages. Thus, stage VII was the most long-lasting, whereas stages II and III were the shortest stages of the cycle of the seminiferous epithelium. No difference in stage frequency distribution was detected between hamsters killed 3 h and hamsters killed 8 days 3 h after BrdU injection. Statistical analysis revealed no significant differences in stage frequencies between the three groups.

Three hours after BrdU administration, most labeled germ cells were observed in stage IX in all hamsters without significant differences between the groups; therefore, all these data were merged and used as reference. The most advanced labeled cells 8 days 3 h after administration were found in most individuals in stage IX, except for one hamster in group 1 (24 h), in which the most labeled germ cells were observed in stage X. Microscopic analyses revealed that 3 h after BrdU injection the most advanced labeled cell type was the primary spermatocytes at the leptotene step in stage IX, whereas 8 days 3 h after administration the most advanced labeled cell type was the pachytene spermatocytes of stage IX. Significant differences in staining frequencies in stage VIII between group 24 h and group 23 h ($P < 0.01$) in hamsters killed 3 h after injection and in stage VII ($P < 0.05$) between group 24 h and group 23 h in hamsters killed 8 days 3 h after injection were found (Fig. 4).

The durations of the cycle of the seminiferous epithelium, calculated from the stage and staining frequencies, showed remarkably small variations and were not different between the groups (24 h: 7.98 $\pm$ 0.05 days; 23 h: 7.94 $\pm$ 0.04 days; 25 h: 7.91 $\pm$ 0.04 days [mean $\pm$ SEM]; $P > 0.05$). The coefficients of variations were small (24 h: 2.0%; 23 h: 1.4%; 25 h: 1.4%). There was no difference in DCSE values between animals that were entrained and those that were not entrained. In the 23-h group, for example, the DSCEs of the eight entrained animals were 7.92 $\pm$ 0.03, vs. 7.94 $\pm$ 0.04 for all 10 animals of that group.

DISCUSSION

The DCSE in Djungarian hamsters as reported herein (7.91 to 7.98 days) is in very good agreement with data published previously (7.9 days) [4]. This comparison shows both that the modified methodological approach had no influence on the results, and that the DCSE in this species that has been kept as a laboratory animal for almost 3 decades [23] is not different in studies that were performed almost 2 decades apart from each other. Also, in a study in feral and laboratory rats, no differences in DCSE were found [5]. It seems therefore reasonable to label the DCSE as a species-specific biological constant.

The assumption that the process of spermatogenesis in mammals is under genetic control is supported by a number of facts. First, the high temporal precision on the order of 1% and its constancy within a species over many generations appears to be incompatible with purely cytological or biochemical processes, especially taking into account the unavoidable errors of the method of determining the DCSE, but points to a process already under genetic control [24]. Second, the DCSE is shorter in immature hamsters and rats [11], which is compatible with the ontogeny of genetic networks of the SCN and the pars tuberalis of the pituitary, as recently shown in mice [25]. Third, *Arntl* knockout mice are infertile, although this may be due to indirect effects, because the levels of testosterone, luteinizing hormone, and follicle-stimulating

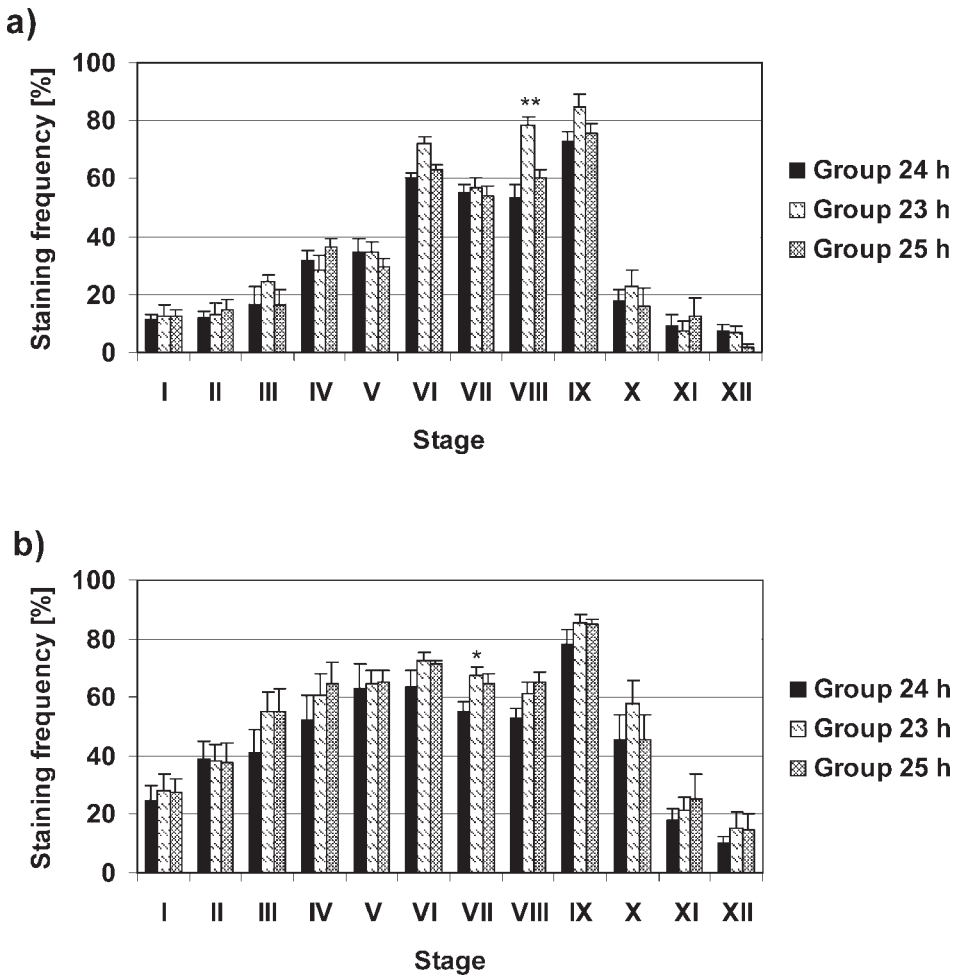

FIG. 4. Staining frequencies of the seminiferous epithelium estimated 3 h **(a)** and 8 days 3 h **(b)** after injection with BrdU. *$P < 0.05$, **$P < 0.01$ vs. 24 h.

hormone were found to be affected as well [26]. The authors of the aforementioned study speculate that the effects may be due to a diminished production of testosterone because the levels of luteinizing hormone were elevated. In fact, they were able to demonstrate diurnal expression of *Arntl* in Leydig cells. This observation corresponds to data showing cyclic (approximately 24 h) expression rhythms in isolated interstitial cells from rat testes; in that study, however, no circadian gene expression was observed in Leydig cells. [16].

One may be tempted to speculate that the high temporal precision of the duration of the spermatogonial cycle is in fact not only under genetic control, but caused by a ''testicular clock,'' thus by another peripheral biological pacemaker. This conclusion, however, is for the time being too speculative. First, the presence of just one gene being expressed in a diurnal fashion does not prove the existence of a full-fledged genetic network of clock genes [27]. Second, the period lengths of circadian clocks (roughly 24 h) are not compatible with the much longer DCSE. It is, however, very interesting to note that the values of DCSE in several hamster species are very close to or even indistinguishable from multiples of 24 h. These values are 7.9 days for Djungarian hamsters [4] (also confirmed in this study), 17.0 days in Chinese hamsters [3], 9.0 days in Syrian (golden) hamsters [28], and 8.0 days in Turkish hamsters [29].

The results obtained with respect to the cyclic expression of *Per1* are in agreement with data obtained in Syrian hamsters [21] but in conflict with results reported for mice [30–32]. This fact may indicate species-specific differences in the testicular expression pattern that deserve to be addressed in further studies. In some studies in mice and hamsters, however, it was found that the expression of *Per1* is restricted to specific stages of spermatogenesis [21, 31]. Another interesting observation is that *Per1* has been found to be expressed in a cyclic fashion in peripheral male reproductive organs, most prominently in the prostate, where the expression patterns of this gene was in antiphase with *Arntl* and showed similar patterns as observed in the SCN [33]. The lack of a prominent oscillatory pattern of *Arntl* in testes of Djungarian hamsters, as reported herein, is in agreement with studies in mice [30–32, 34], but in contrast to a study in Syrian hamsters [21], again indicating possible species-specific differences.

The approach of this study was to test whether the stunningly precise process of spermatogenesis can be altered by forcing an entrainment to shortened or prolonged T-cycles and thereby synchronizing the entrained central pacemaker (the SCN) with spermatogenesis in the testis. That peripheral oscillators, even within the same organ, can be dependent or independent of the central pacemaker has been shown in mice [27]. Other studies in mice have shown that the electrolytic lesion of the SCN eliminates the oscillations of *Per1* and *Arntl* in the mouse liver [35]. Thus, if the temporal organization of spermatogenesis were dependent on the entrained central pacemaker (to a 23- or 25-h T-cycle), a difference of approximately 4.2% in DCSE would have resulted. Assuming equal variances, this difference, despite the small absolute values, would have been highly significant. To illustrate this important point, an example is given. The DCSE of animals exposed to 24-h day length was 7.98 $\pm$ 0.05 days. If the DCSE

were dependent on and synchronized by the central pacemaker, a value for DCSE of 7.64 ± 0.05 would have resulted in the hamsters exposed to a T-cycle of 23 h. This value would have been highly significantly different from the DCSE of the control group ($P < 0.001$). That this difference was not found, and not even a tendency in this direction was found, allows us to conclude that the temporal pattern of spermatogenesis is independent from the central clock. The fact that two significant differences were found in the staining frequencies in stages VII and VIII (Fig. 4) may indicate that, despite the ineffectiveness of altered T-cycles to change the DCSE, some effects on spermatogenesis may have occurred. We do not, however, put too much emphasis on these significant differences because they may have occurred by chance because of the high number of statistical comparisons.

One limitation of the study is the fact that most of the animals exposed to 25-h day lengths were not entrained throughout the experimental period. It therefore seems that this species is less suited to be entrained to altered day lengths compared to other species [18]. Nevertheless, the data obtained from the fully entrained animals exposed to the T-cycle of 23 h are sufficient for the principal conclusion of our study.

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