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Research Report

Circadian modulation of interval timing in mice

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ABSTRACT

Temporal perception is fundamental to environmental adaptation in humans and other animals. To deal with timing and time perception, organisms have developed multiple systems that are active over a broad range of order of magnitude, the most important being circadian timing, interval timing and millisecond timing. The circadian pacemaker is located in the suprachiasmatic nuclei (SCN) of the hypothalamus, and is driven by a self-sustaining oscillator with a period close to 24 h. Time estimation in the second-to-minutes range – known as *interval timing* – involves the interaction of the basal ganglia and the prefrontal cortex. In this work we tested the hypothesis that interval timing in mice is sensitive to circadian modulations. Animals were trained following the peak-interval (PI) procedure. Results show significant differences in the estimation of 24-second intervals at different times of day, with a higher accuracy in the group trained at night, which were maintained under constant dark (DD) conditions. Interval timing was also studied in animals under constant light (LL) conditions, which abolish circadian rhythmicity. Mice under LL conditions were unable to acquire temporal control in the peak interval procedure. Moreover, short time estimation in animals subjected to circadian desynchronizations (modeling jet lag-like situations) was also affected. Taken together, our results indicate that short-time estimation is modulated by the circadian clock.

1. Introduction

Timing and time perception are fundamental to survival and goal reaching in humans and other animals. Organisms have developed diverse mechanisms for timing across different scales, the most important being circadian timing, interval timing and millisecond timing (Buhusi and Meck, 2005). The circadian pacemaker – which is driven by a self-sustaining oscillator with a period close to 24 h – is located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Dunlap et al., 2004), and the principal signal that adjusts its activity is the light–dark cycle (Morin and Allen, 2006; Golombek and Rosenstein, 2010). The molecular mechanism of the endogenous circadian clock is comprised by interlocked transcription-translation feedback loops (Reppert and Weaver, 2002). On the other hand, the perception of shorter durations in the seconds-to-minutes range, known as interval timing, is crucial to learning, memory, decision making and other cognitive

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tasks. Recent findings argue for the involvement of cortico-striatal circuits that are controlled by the dopaminergic modulation of oscillatory activity and lateral connectivity. Striatal medium spiny neurons detect the coincident activity of specific beat patterns of cortical oscillations (Matell and Meck, 2004; Meck, 2006).

Current evidence suggests that the pacemaker or internal clock which mediates the perception of short durations is sensitive to temperature, attention, emotions, drug and diet manipulations (e.g., Wearden and Penton-Voak, 1995; Cheng et al., 2006; Droit-Volet and Meck, 2007), all of which can be modulated by circadian rhythms. Although the suprachiasmatic nuclei appear to be unnecessary for interval timing (Lewis et al., 2003), time of day effects have been observed for the timing of auditory and visual signals in the seconds-to-minutes range (Aschoff, 1985; Chandrashekaran et al., 1991; Pati and Gupta, 1994; Meck, 1991). For example, several studies have shown that time judgments in humans covary with normal circadian rhythms. (e.g., Kuriyama et al., 2005; Lustig and Meck, 1991). Consistent with this finding, a circadian rhythm in time estimates was documented in control subjects, but it was found to be disrupted in shift workers (Pati and Gupta, 1994). Moreover, rats exhibit circadian variations in time perception similar to those that have been demonstrated in humans (Shurtleff et al., 1990). Furthermore, it was recently reported that sleep deprivation influences diurnal variation of time estimation in humans (Soshi et al., 2010). In *Drosophila melanogaster*, timing of short intervals is disrupted in circadian mutants (Kyriacou and Hall, 1980).

The present work was designed to study the role of the circadian system in the modulation of time estimation in the seconds-to-minutes range. To determine whether time of day influences interval timing, animals were trained using a 24-s peak-interval (PI) timing procedure at different times of day. The PI procedure (Roberts, 1981) is one of the tasks most frequently employed in the research on timing behavior (reviewed in Matell and Meck, 2004). In this task, two types of trials are randomly alternated: fixed interval (FI) trials and peak interval (PI) trials. In FI trials, a discriminative stimulus is presented, and the first response after a time interval has elapsed is followed by the delivery of a reward. In peak trials, the same discriminative stimulus is presented for a duration two to three times longer than the FI, and the reward is never delivered. Averaging across these "probe" trials produces a Gaussian-shaped response function that peaks very close to the criterion time. Representative parameters – peak height, peak width, and peak location – are obtained by fitting these Gaussian curves. In this work we also analyzed the acquisition of both S1 (start responding) and S2 (stop responding) thresholds (Church et al., 1994; Gallistel et al., 2004). These thresholds are proposed to be indices of temporal behavior and can be selectively altered by different treatments (e.g., Gooch et al., 2007).

Our results indicate that time estimation in mice is more accurate at night than during the day. In addition, interval timing was studied in animals under constant light (LL) conditions, which abolish circadian rhythmicity. Mice trained under LL conditions were unable to acquire reliable temporal control of lever pressing in the PI procedure. Moreover, short time estimation in animals subjected to a 6-h advance of the light/dark cycle was transiently affected.

2. Results

2.1. Diurnal and circadian rhythm in interval timing

In order to investigate daily differences in time perception, mice under a 12 h:12 h light–dark cycle (LD conditions) were trained either in the middle of their diurnal phase at Zeitgeber Time (ZT) 4–6 or in the middle of their nocturnal phase at ZT15–17. Both groups received ten lever-press training sessions to learn to consume the liquid reward, and there were no differences in the speed (number of sessions) with which this response was acquired [$F(1,12)=0.87$, $p>0.05$, two-way repeated measures ANOVA] nor the number of total lever presses [$t(12)=0.93$, $p>0.05$, two-tailed t-test, $n=7$/group].

The response rate during fixed-interval (FI) training was analyzed in both groups for the 24-s interval. All mice acquired a temporal control for this signal duration across sessions (15 FI sessions). However, mice trained during the day were less efficient in their ability to acquire this response relative to the group trained at night. The S1 ("start") rate index was calculated by taking the response rate in a specified interval just prior to the observed peak time as a ratio of overall response rate within the first part of the trial (see Experimental procedures). There was a significant effect of the LD cycle on the S1 rate index during the last 3 sessions of training, but not at the beginning of the sessions. Although a two-way repeated measures ANOVA comparing the S1 rate index across sessions blocks indicated a non-significant effect of group [$F(1,12)=1.87$, $p>0.05$], there was a clear trend for the S1 rate index to be higher for the group trained during the night. Moreover, a comparison of the S1 rate index during the last 3-session block (sessions 13–15) indicated a significant difference for mice trained during the day or night [$t(12)=3.69$, $p<0.01$, two-tailed t-test, $n=7$/group].

Peak-interval (PI) training was used to evaluate the accuracy and precision of the S2 ("stop") response for the 24-s target duration. The mean proportion of maximum response rate plotted as a function of time for the last session block (sessions 21 to 24) is shown in Figs. 1A and B for night- and day-trained mice, respectively. Both groups gradually learned to stop responding after the criterion time, reaching a Gaussian-shaped response function. However, there were differences in the parameters of this function, as illustrated in Figs. 1C–E. Compared to the group trained at night, in the day-trained group peak location was significantly increased [right-shifted, $t(12)=2.29$, $p<0.05$], peak height was reduced [$t(12)=2.36$, $p<0.05$] and peak width was significantly increased [$t(12)=3.40$, $p<0.01$] (two-tailed t-test for the last session block of PI training in all cases, $n=7$/group). Moreover, there were significant differences in the mean S2 ("Stop") rate index in both groups during the last sessions of PI training. The S2 rate index was calculated by taking the response rate in a specified interval just after the observed peak time as a ratio of overall response rate within the rest of the trial after criterion time (see Experimental procedures). As shown in Fig. 1F, the S2 rate index was higher in the nocturnal group [$F(1,12)=10.97$, $p<0.01$, two-way repeated measures ANOVA], suggesting a better performance in this timing task during the night.

Importantly, this difference also continued in constant darkness (DD), demonstrating the endogenous nature of this

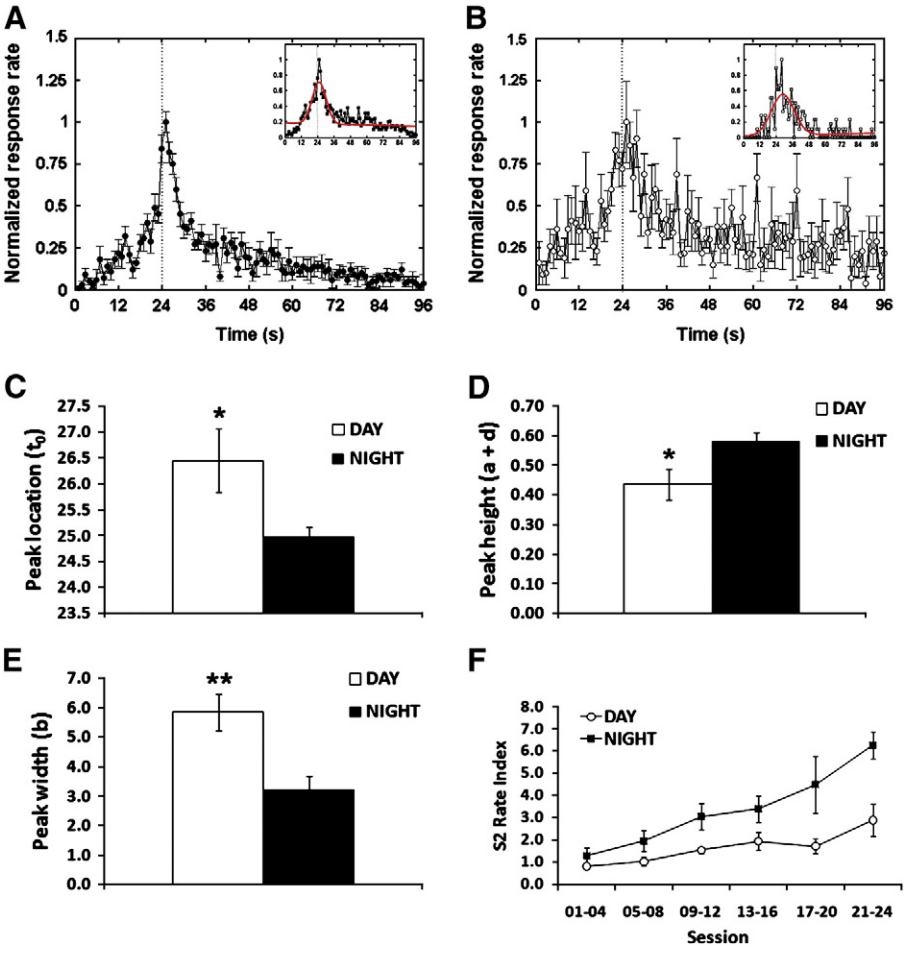

Fig. 1 – Effect of LD cycle. Normalized response rate of PI trials as a function of time in the trial in mice trained and tested either during the middle of the night at ZT 15–17 (A) or during the middle of the day at ZT 4–6 (B) during the last 4-session block (sessions 21–24) of PI training. Insets in panels A and B correspond to an example of curve fitting (straight line) to the experimental data. (C) to (E) show the mean best-fit parameter values from the Gaussian fits from last session block. (C) Peak location. (D) Peak height. (E) Peak width. (F) Represents the S2 rate index. Data are shown as mean ± S.E.M. (n = 7/group). Best-fit parameter values: $^{**}p < 0.01$, $^{*}p < 0.05$, two-tailed t-test. S2 Index: $p < 0.0001$ for sessions; $p < 0.01$ for groups (two-way repeated measures ANOVA).

modulation (Supplementary Fig. 1). In DD conditions, peak width and S2 rate index showed significant differences between groups. Thus, in the subjective night-trained group peak width was lower [$t(10) = 2.35$, $p < 0.05$] while S2 rate index was higher [$F(1,10) = 40.31$, $p < 0.001$], indicating that the better performance observed during the night was maintained in constant dark conditions. Peak location and peak height continued with the same tendency that was observed in LD (i.e., a better performance during the subjective night) but this difference was not quite significant [$t(10) = 1.84$, $p > 0.05$ and $t(10) = 1.91$, $p > 0.05$, respectively, n = 6/group]. Table 1 summarizes all parameters for the diurnal and circadian rhythms in interval timing.

Table 1 – Interval timing parameters during PI training in LD and DD conditions [a]

Parameters	LD conditions		DD conditions	
	Day	Night	Subjective day	Subjective night
Peak position (t_0) [b]	26.45 ± 0.61	24.99 ± 0.18	27.72 ± 1.05	25.81 ± 0.40
Amplitude (a) [b]	0.43 ± 0.05	0.58 ± 0.08	0.40 ± 0.02	0.57 ± 0.08
Width (b) [b]	5.84 ± 0.63	3.24 ± 0.43	6.89 ± 1.01	3.69 ± 1.01
S2 rate index [c]	2.89 ± 0.74	6.26 ± 0.62	2.66 ± 0.17	4.25 ± 0.36

[a] Data are expressed as mean ± S.E.M. LD: n = 7/group; DD: n = 6/group.
[b] Mean best-fit parameter values from the Gaussian fits during the last 4-session block (sessions 21–24) of PI training.
[c] Corresponding to the last 4-session block (sessions 21–24) of PI training.

2.2. *Effect of LL conditions*

Constant light (LL) conditions induce period lengthening followed by circadian arrhythmicity in mice (e.g., Meng et al., 2010). In order to study the effect of circadian arrhythmicity on interval timing, mice were switched to LL conditions (100 lx) for at least 25 days before being subjected to the PI protocol. While mice that were entrained to a 12 h:12 h light/dark cycle (LD) exhibited robust wheel running activity rhythms, mice under constant light conditions (LL) became arrhythmic (Supplementary Figs. 2A and B, respectively).

Circadian arrhythmicity under constant light conditions did not impair acquisition of lever pressing, since there were no differences in the number of presses [$t(14)=1.33$, $p>0.05$, two-tailed t-test] nor the speed (number of sessions) with which lever press training was acquired [$F(1,14)=1.76$, $p>0.05$, two-way repeated measures ANOVA, n=8/group].

During the FI procedure, mice trained under the LD cycle (ZT 15–17) demonstrated an increase of temporal control along sessions similar to our previously observed results. In contrast, the group trained under LL conditions failed to exhibit temporal control of their responses after 18 sessions of 24-s FI training [$F(1,14)=19.32$, $p<0.001$, two-way repeated measures ANOVA, n=8/group].

During PI training, the group of mice under constant light continued to exhibit a total loss of temporal control, as illustrated in Fig. 2. The mean proportion of maximum response rate plotted as a function of time for the last session block (sessions 21 to 24) is illustrated in Figs. 2A and B for the LD and LL groups, respectively. Mice maintained under LL conditions were disrupted in the performance of the PI procedure and never produced the typical Gaussian-shaped mean response function. Moreover, there were significant differences in the mean S2 rate index in both groups during the last sessions of PI training [$F(1,14)=21.11$, $p<0.001$, two-way repeated measures ANOVA, n=8/group], as shown in Fig. 2C.

In order to assess possible visual impairments in mice under LL conditions, scotopic electroretinograms were performed. Both a and b-wave amplitudes and latencies were normal in mice under LD or LL conditions (Supplementary Figs. 2C and D), ruling out visual alterations in this group.

2.3. *Effect of an abrupt change of the LD cycle*

Mice that had been successfully trained to the PI protocol under light/dark conditions (n=7) were subjected to a 6-h advance of the LD cycle (jet-lag simulation) at the end of session # 28 of PI training. After this 6-h advance, animals received 20 additional training sessions. A control group (n=4) received the same amount of sessions without any change in the light/dark cycle. In mice subjected to the 6-h advance, the circadian rhythm of running-wheel activity was gradually resynchronized to the new light regimen. Complete resynchronization was acquired after 8–9 days (data not shown). Interval timing in these animals was significantly affected by the light schedule change, as shown in Fig. 3A. In the 4-session block following phase advance, there was a significant change in the Gaussian parameters. Compared to the session block previous to the 6-h advance (B0), in the session block

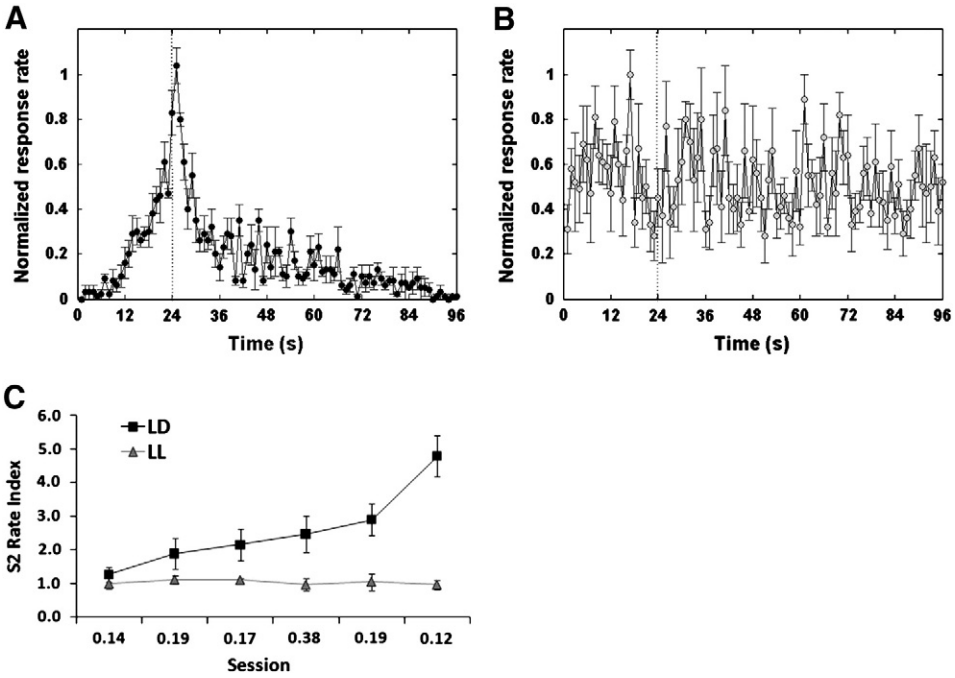

Fig. 2 – Effect of LL conditions on interval timing. Normalized response rate of PI trials as a function of time in the trial in mice trained and tested either during LD at ZT 15–17 (A) or during LL (B) conditions. The last 4-session block (sessions 21–24) of PI training is shown. (C) S2 rate index under LD or LL conditions. Data are shown as mean ± S.E.M. (n=8/group). $p<0.0001$ for sessions; $p<0.0001$ for groups (two-way repeated measures ANOVA).

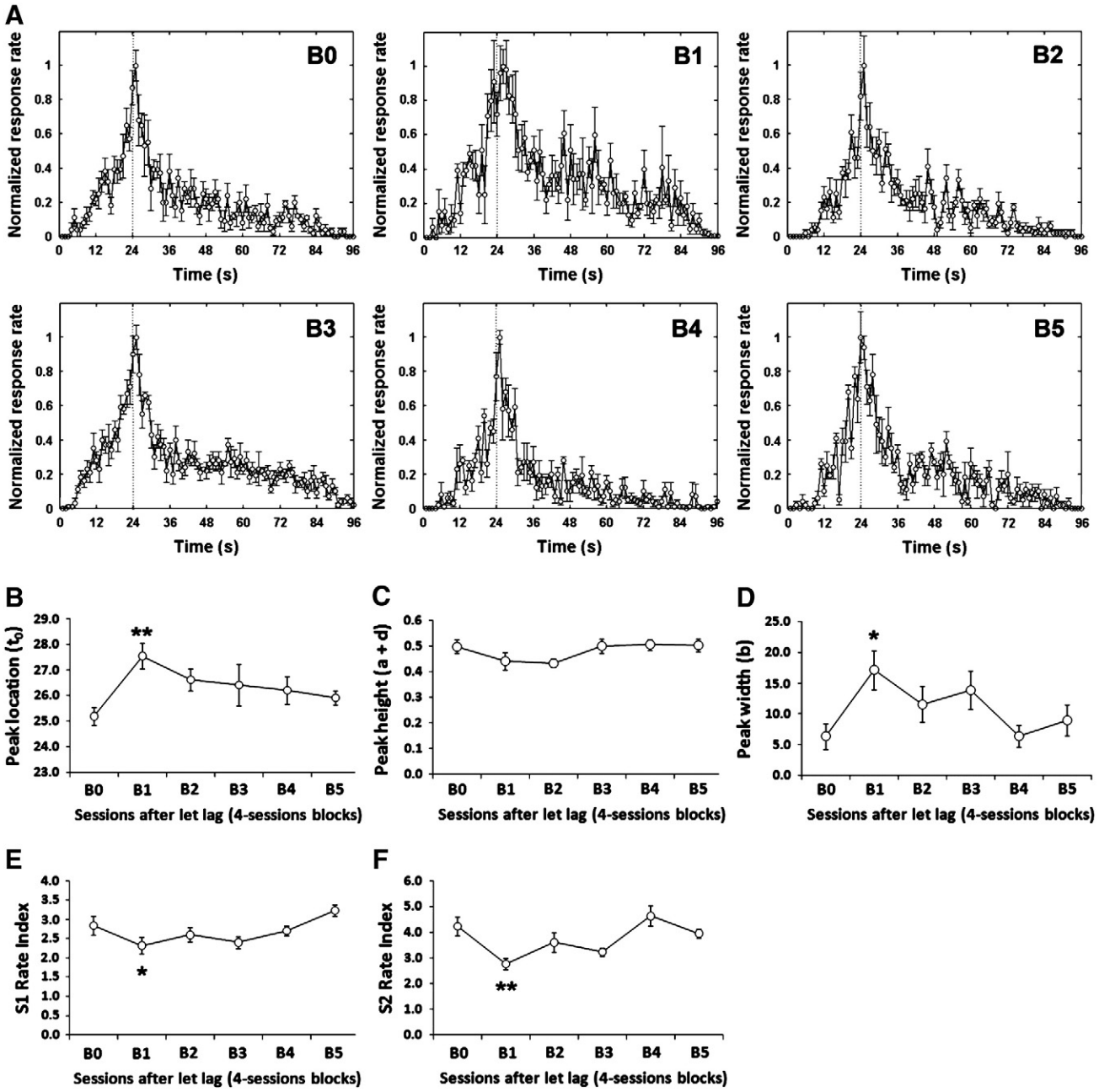

Fig. 3 – Effect of jet-lag simulation on interval timing. (A) Normalized response rate of PI trials as a function of time in the trial in mice subjected to a 6-hour advance of the LD cycle. Each plot represents 4-session blocks. B0 indicates the 4-session block previous to the 6-hour advance of the LD cycle; B1 to B5 indicate consecutive 4-session blocks after the advance. (B) to (D) show the mean best-fit parameter values from the Gaussian fits. (B) Peak location. (C) Peak height, (D), Peak width. (E) and (F) show the S1 and S2 rate indexes, respectively. Data are shown as mean ± S.E.M. (n = 7/group). $^{**}p < 0.01$, $^{*}p < 0.05$ (repeated measures ANOVA followed by Tukey test).

immediately following the phase advance (B1) there was a significant increase in peak location [$F(5,30) = 3.029$, $p < 0.05$] and width [$F(5,30) = 2.54$, $p < 0.05$] and a tendency to decrease in the amplitude [$F(5,30) = 1.88$, $p > 0.05$] of the Gaussian function (repeated measures ANOVA followed by Tukey's test, Figs. 3B–D), denoting a lower efficiency of interval timing at this stage. Moreover, there was a significant decrease in the mean S1 and S2 rate indexes immediately following change of the LD cycle [$F(5,30) = 4.91$, $p < 0.01$ and $F(5,30) = 6.86$, $p < 0.001$, respectively, Figs. 3E and F]. All parameters returned to basal levels (i.e., similar to the ones previous to the advance) within 6–8 sessions, indicating a transient effect that could be related to the negative effects of this jet-lag simulation. Moreover, there were no significant changes in these parameters in the

control group (data not shown). Taken together, these data indicate that a temporal desynchronization of the circadian system is able to negatively affect time estimation in mice.

3. Discussion

In mammals, the mechanism for the generation and entrainment of circadian rhythms resides in the hypothalamic suprachiasmatic nuclei (SCN), and the principal signal that adjusts this biological clock with environmental timing is the light–dark cycle. Under conditions of continuous darkness, circadian rhythms assume free-running periods that are close to 24 h. In this study, we investigated the circadian influence in the acquisition of temporal learning in mice trained using the PI procedure. Our results show that mice performed better when they were trained in the nocturnal phase of the light/dark cycle, as well as in their subjective night when assessed under constant dark conditions. These results are in agreement with previous work related to circadian modulation of other behavioral and physiological functions such as synaptic plasticity. For example, Chaudhury et al. (2005) demonstrated in mice a diurnal rhythm in excitability and LTP in hippocampal brain slices, with an enhancement of the population spike magnitude in LTP recorded from subjective night slices compared to subjective day slices.

Circadian rhythms are disrupted under constant light (LL) illumination. Nocturnal rodents in LL exhibit reduced locomotor activity and an unusually long circadian period, and at high light intensities they become arrhythmic. Our results indicate that circadian arrhythmicity under constant light conditions impaired their ability to properly estimate a 24-s interval. Notably, previous studies have reported different results for the circadian modulation in interval timing. In particular, Lewis et al. (2003) suggest that both systems are independent since they found no effects of suprachiasmatic nuclei lesions on interval timing mechanisms. However, the extent of the lesions is not indicated in their work. These authors also stated that the relatively poor curve fitting on interval timing – which occurred during DD – could not be explained either as a response to the lesion or circadian arrhythmicity. Additional experiments regarding SCN lesions need to be performed to clarify this issue.

On the other hand, jet-lag evokes a transient desynchronization of the circadian system. After an abrupt change of the light/dark cycle, circadian rhythms gradually adapt to the new environmental conditions. Thus, resynchronization to a 6-h advance of the LD cycle usually takes 8–10 days in rodents (Agostino et al., 2007; Kiessling et al., 2010). In the present work we show that this phase shifting process transiently affected interval timing in mice. Indeed, there was a change in all the parameters studied in the sessions immediately following the phase advance, which gradually returned to levels similar to the ones previous to the shift. This transient effect on time estimation could be related to the negative effects of a jet-lag simulation. It was recently reported that the process of jet-lag is characterized by marked heterogeneity of phase resetting of specific genes that operate in the positive and negative branches of the circadian clock (Kiessling et al., 2010). In this sense, misalignment of the transcriptional feedback loops driving the circadian molecular clock may be involved in the transient perturbation of short time estimation. Moreover, specific processes which require neural plasticity, such as a variety of learning and memory procedures, are also affected by circadian manipulations that include changes in the LD cycle (Cain et al., 2004), suggesting that circadian desynchronization transiently impair several cognitive mechanisms, although the exact mechanism through which this occurs is currently not understood.

Although the use of a single target duration (24-s) may have some limitations, such as the possibility to investigate proportional effects (e.g., scalar property), our results indicate that, at least for this particular interval, animal responses were not independent from circadian changes. Indeed, interval and circadian timing might share some common features related to their molecular mechanisms and might also influence one another. This regulation can be interpreted in terms of adaptation requirements, since a variable accuracy of time estimation might be needed at all times throughout the daily cycle.

In addition, both timing mechanisms might be affected by neural circuits regulating motivational state. For example, acquisition of operant cycles of reinforcement requires the activation of reward pathways in the brain, usually driven by food stimulation in partially deprived animals. It has been shown that restricted daily food access acts as an entraining stimulus for an SCN-independent circadian clock, the feeding-entrainable oscillator (FEO). Food-entrained circadian rhythms are characterized by increased locomotor activity in anticipation of food availability (food anticipatory activity, FAA). In our experimental protocol, food was given after each experimental session. In mice trained during the night, daily feeding occurred during the nocturnal period, when the animals are active; in this situation no FAA is predicted. Although actograms from day-trained mice appear to be less robust, locomotor activity is still entrained to the light–dark cycle, without any FAA. It is possible that the use of a sweetened solution and not a food pellet, contributes to the fact that in our experiments feeding was not interpreted as a Zeitgeber. In addition, it should be stated that liquid reward, rather than food, may have contributed to the relatively low levels of lever pressing shown in our results.

It is interesting that at least some features of circadian entrainment (such as nonphotic synchronization) also rest upon reward-related mechanisms of the brain, which might include dopaminergic activation (Webb et al., 2009). A common dopaminergic (among other neurochemical pathways) background might link some features of timing mechanisms in the brain.

As for common molecular effectors, Roybal et al. (2007) indicated that the central transcriptional activator of molecular rhythms, CLOCK, has an important role in the ventral tegmental area (VTA) in regulating dopaminergic activity, locomotor activity, and anxiety. Moreover, several genes involved in dopaminergic signaling are differentially regulated in the VTA of the *Clock* mutant mice, suggesting that CLOCK affects the transcription of these genes through its actions in this brain region. In this sense, the *clock* gene appears to be a common step in both timing mechanisms, at two very different time scales.

However, it was reported that, when housed under a light/dark cycle, the *Clock* mutant mice have no reliable deficits in the accuracy or precision of short time estimation (Cordes and Gallistel, 2008). It should be noted that under these conditions *Clock* mice entrain to the LD cycle and maintain rhythmicity like their wild-type littermates. In complete darkness, however, *Clock*–/– mice first express abnormal periods and later become completely arrhythmic (Vitaterna et al., 1994). In this sense, it would be interesting to study the effect of *Clock* mutation on interval timing under constant dark conditions. In addition, other circadian genes need to be explored in order to search for the molecular bases of these two mechanisms (e.g., *Period*).

The SCN is able to influence reward-related mechanisms by a circadian modulation of dopaminergic neurotransmission within mesolimbic structures. Indeed, dopamine-related substances, metabolites and receptors exhibit daily fluctuations in their levels in different brain regions (Kafka et al., 1986). Moreover, many brain regions implicated in reward-seeking behavior also contain "peripheral" (as opposed to SCN-driven) molecular clocks. Circadian fluctuations in extracellular dopamine levels in the striatum and nucleus accumbens have been described (Castaneda et al., 2004). Furthermore, identification of specific clock binding elements (E-boxes) within the promoter regions of the dopamine transporter, dopamine D1A receptor, and tyrosine hydroxylase genes (Kawarai et al., 1997; Weber et al., 2004) supports the existence of an interaction between circadian clocks and dopaminergic neurotransmission. Indeed, it was discovered that the SCN is at least partially responsible for the presence of normal day/night differences in dopamine transporter and tyrosine hydroxylase protein expression in the nucleus accumbens, mPFC, and caudate (Sleipness et al., 2007b). A contribution of the SCN in the day/night variation in cocaine-seeking behavior in rats has also been reported (Sleipness et al., 2007a). It is interesting to consider that these rhythms might share a common ground with learning and memory processes whose methodological considerations include a reward step necessary for the establishment of the behavior. Several lines of research have indicated a circadian rhythm for diverse phases of learning and memory in different animal models, including rodents and humans (e.g., Eckel-Mahan and Storm, 2009).

In summary, our data suggest that the ability to temporally control responding in the PI procedure is not independent from the circadian system. Although the mechanisms and specificity by which the biological clock regulates interval timing are not known, there are some potential candidates that should be explored in future studies. Among them, the study of common dopaminergic pathways as well as clock gene expression in extra-SCN brain areas will shed more light on the regulation of timing behavior.

4. Experimental procedures

4.1. Animals

Mice (C57BL6) were purchased from commercial suppliers (Bioterio Central, Universidad Nacional de La Plata) and were maintained in a 12 h:12 h light–dark cycle (LD, lights on at 0600 h) with food and water *ad libitum* and room temperature set at 20±2 °C. Male adults (3–4 month-old) animals were used throughout the experiments. When animals had to be handled in the dark, we used a dim red light source (<5 lx). Mice were maintained at 90% of their *ad lib* weight by restricting access to food. All animal procedures were performed in strict accordance with NIH rules for animal care and maintenance.

4.2. Experimental groups

In experiments carried under light/dark conditions (LD), mice were trained either in the middle of their diurnal phase at Zeitgeber Time (ZT) 4–6 or in the middle of their nocturnal phase at ZT 15–17. By convention, ZT 12 is defined as the time of lights off. Under constant dark conditions (DD), animals were trained at Circadian Time (CT) 4–6 or CT 15–17. By convention, CT 0 is defined as the onset of activity. Experiments under constant light conditions (LL) were carried out at the same clock hours as their LD controls. For jet-lag simulation experiments, mice were subjected to an abrupt 6-h advance in the phase of the LD cycle. Clock time for training and testing was the same, even after the advance of the LD cycle, That is, animals changed their LD cycle but there was no shift in the timing of the interval timing task, which was always in the diurnal phase (ZT8–10 before and ZT2–4 after the 6-h advance).

4.3. Activity rhythm recording

Animals were transferred to individual cages equipped with a running wheel (7 cm. diameter) and with light intensity averaging 200 lx at cage level. Running-wheel activity was continuously recorded for each animal using a digital system that registers wheel revolutions and stored at 5-min intervals for further analysis. Animals were maintained under a 12 h:12 h LD cycle. In mice that were subjected to an abrupt 6-h advance in the phase of the LD cycle, time for reentrainment to the new LD cycle was defined as the time it took for each animal – expressed in days – to achieve its activity onset into the new cycle. Resynchronization was considered fully accomplished when each animal's activity onsets took place at the new time of lights off±15 min. For constant light experiments (LL), animals were continuously exposed to light (100 lx) for at least 25 days before the interval timing training. For constant dark experiments (DD), mice were entrained in 12 h LD cycles and then released into DD conditions for at least 10 days before training.

4.4. Electroretinography

Electroretinographic activity was assessed in dark-adapted rodents as previously described (Moreno et al., 2005). Briefly, after 6 h of dark adaptation, mice were anesthetized with an intraperitoneal injection of ketamine hydrochloride and xylazine hydrochloride under dim red illumination. Phenylephrine hydrochloride (2.5%) and 1% tropicamide (Alcon Laboratories, Argentina) were used to dilate the pupils, and the cornea was intermittently irrigated with balanced salt solution (Alcon Laboratories, Argentina) to maintain the baseline recording

and to prevent exposure keratopathy. All recordings were completed within 20 min of the induction of anesthesia. Electroretinograms (ERGs) were recorded from both eyes simultaneously and ten responses to flashes of unattenuated white light (5 ms, 0.2 Hz) from a photic stimulator (light-emitting diodes) set at maximum brightness (9 cd s/m^2 without a filter) by a full-field Gandzfeld, were amplified, filtered (1.5-Hz low-pass filter, 1000 high-pass filter, notch activated) and averaged (Akonic BIO-PC, Akonic, Argentina). The a-wave was measured as the difference in amplitude between the recording at onset and the trough of the negative deflection and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. Electrophysiological responses were averaged for each run. Runs were repeated 3 times with 5-min intervals, and the mean of these 3 runs was used for subsequent analysis. We compared the mean peak latencies and peak-to-peak amplitudes of the responses from each group of mice.

4.5. *Interval timing protocol*

4.5.1. *Apparatus*

Experimental chambers (internal dimensions 30×22×14 cm) were designed at the investigators' laboratory. Each chamber was located in a light- and sound-attenuated cabinet equipped with a fan, which provided background white noise inside the chamber. Chambers were equipped with one retractable lever situated on the front wall of the box. According to the schedule, a reward of one drop of water with 5% of sucrose was provided by pressing the lever, which was mounted on the same wall as the reward delivery, 5 cm away and 3 cm above the floor. For the FI and PI training, the stimulus was a 50-lux house light mounted at the center-top of the front wall.

Animals were trained following the peak-interval (PI) procedure in three consecutive phases — lever press training, fixed interval training and peak interval training (Cheng and Meck, 2007; Drew et al., 2007). In all segments of the experiment, sessions occurred once per day, 5 days per week.

4.6. *Lever-press training*

Mice were trained to drink the liquid reward by pressing the lever. At the beginning of the session, the lever was extended into the chamber, and lever presses were reinforced on a continuous reinforcement schedule. In this and all subsequent sessions, the reward consisted of one drop of water with 5% sucrose. To familiarize mice with the retraction and extension of the lever, it was retracted after the 20th reinforcement. After a variable delay, the lever was extended, and the cycle was repeated. After five days with this protocol, mice received a shorter continuous reinforcement training session. The session began with the lever extended. The lever was retracted every two reinforcements and then reextended after a variable intertrial interval. The session ended when the mouse earned 60 reinforcements or 1 h elapsed. After another five days of this kind of sessions, mice moved to fixed interval (FI) training.

4.7. *Fixed-interval (FI) procedure*

A visual signal (50 lx) was used to time a fixed interval. Lever presses were not reinforced until after this interval had elapsed. Mice received a FI 24-s schedule, meaning that the first lever press 24-s after the beginning of the signal triggered the delivery of a drop of reward and terminated the visual signal for the duration of the random intertrial interval (ITI). Trials were separated by a 10–110-s uniformly distributed random ITI. Session duration was 60 min. All animals received at least 15 FI sessions.

4.8. *Peak-interval (PI) procedure*

After the FI training, mice received 24 sessions of PI training, as follows. During each session, animals received 50% FI trials randomly intermixed with 50% non-reinforced probe trials in which the to-be-timed signal remained active three times longer than the FI time, that is, 96-s, before being terminated. Peak trials and FI trials were ordered randomly, with the restriction that no more than five peak trials could occur consecutively. Trials were separated by a 10–110-s uniformly distributed random ITI. Session duration was 90 min. All animals received 24 PI sessions.

4.9. *Data analysis*

Data were used to estimate the peak time, peak rate, and precision of timing from the response functions for each mouse. The number of responses (in 1-s bins) was averaged daily over trials, to obtain a mean response rate for each mouse. Data were fit using a modified Gaussian function (Buhusi et al., 2005), Briefly, mean response-rate functions for the interval of interest were fit using the Marquardt–Levenberg iterative algorithm to find the coefficients (parameters) of a Gaussian+linear equation that gave the best fit (least squares minimization) between the equation and the data. The following Gaussian+linear model was used: $R(t)=a\times\exp(-0.5\times[(t-t_0)/b]^2)+c\times(t-t_0)+d$, where t is the current time, and R(t) is the mean number of responses at time t. The iterative algorithm provided parameters a, b, c, d and t_0. Parameter t_0 (peak location) was used as an estimate of the daily peak time of responding, a+d (peak height) was used as an estimate of the peak rate of response, and parameter b (peak width) was used as an estimate of the precision of interval timing. All the response functions were separately fitted by linear functions and by Gaussian+ramp functions (Buhusi and Meck, 2009). The ratio of the r^2 values from the linear and Gaussian+ramp function was used as a measure of the temporal control exhibited by each mouse. If this ratio was greater than 0.8, the animal's data for that criterion duration was not used. The S1 and S2 rate indexes were determined as previously described (Cheng and Meck, 2007). In the present work, the S1 rate index for the PI 24-s procedure was defined by the response rate occurring during the 3-s period just prior to the observed peak time (i.e., seconds 22–24) divided by the overall response rate for the first 24-s of the trial (i.e., seconds 0–24). Similarly, the S2 rate index was defined by the response rate occurring during the 3-s period just after the observed peak time (i.e., seconds 24–26) divided by the overall response rate during the last 72-s of the trial (i.e., seconds 24–96). Normalized response rates were obtained by first dividing each individual curve by the maximum average response rate, and again, once averaged across individuals, by the maximum response rate

of the average response curve. All statistical tests were evaluated at a significance level of 0.05.

Supplementary materials related to this article can be found online at doi:10.1016/j.brainres.2010.11.029.

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