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Melatonin Modulates Interval Timing in Rats: Effect of Pinealectomy

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Interval timing is a complex cognitive process that involves the estimation of time within the seconds-to-minutes range. This temporal processing depends on cortico-striatal interactions, as well as an optimal dopaminergic function. On the other hand, the circadian system controls physiological and behavioral functions with periods close to 24 hr. We have previously reported that short-time perception in mice is influenced by the circadian pacemaker, with dopamine signaling as a link between both temporal systems. In this work we evaluated the involvement of melatonin in the circadian modulation of interval timing, as well as the interaction between this hormone and dopamine levels in the striatum. We report that melatonin-depleted rats, by pinealectomy, present an impairment in their ability to estimate a short (24 s) target duration in the peak-interval procedure. Moreover, melatonin administration in drinking water restores interval timing precision in pinealectomized rats. We also show that circadian desynchronization causes a transient impairment in the timing task. In addition, melatonin administration affects interval timing only when rats are trained and tested during the night. Furthermore, we report that melatonin depletion increases striatal dopamine availability, which is reverted by external melatonin administration. Taken together, our findings add further support to the notion that the circadian system modulates interval timing, probably by using melatonin as an output to regulate dopaminergic functions in brain areas that are important for interval timing mechanisms.

Circadian rhythms regulate physiological and behavioral functions with periods close to 24 hours (Bass & Takahashi, 2010; Dunlap, Loros, & DeCoursey, 2004). The circadian system is synchronized to daily variations in the environment by cyclic temporal cues. While light is the main synchronizer of the mammalian circadian clock, there are many other signals, both exogenous and endogenous, that can provide temporal information. Melatonin (MT) is released by the pineal gland under the influence of the circadian system. Administration of this hormone, by subcutaneous injection, infusion or via drinking water, is able to synchronize locomotor activity rhythms under constant light or darkness conditions, and accelerates reentrainment to phase shifts in the light-dark schedule (Armstrong, Cassone, Chesworth, Redman, & Short, 1986; Redman, 1997). In both diurnal and nocturnal species, MT release is restricted to nighttime, reaching a peak in the circulating system around Zeitgeber time (ZT) 16-22 in rats (Buijs, van Eden, Goncharuk, & Kalsbeek, 2003). MT receptors are present in several brain structures, and differences in the anatomical distribution patterns of the MT1 and MT2 receptor subtypes suggest different roles for MT signaling

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in such areas (Lacoste et al., 2015). There is evidence that MT correlates with the release of diverse neurotransmitters (Meng, Yuan, Zheng, Liu, & Lin, 2015; Monnet, 2002; Zisapel, 2001). In particular, MT mediates the inhibition of dopamine (DA) release in the hypothalamus, hippocampus and retina (Doyle, Grace, McIvor, & Menaker, 2002; Zisapel, 2001). Moreover, MT controls the daily rhythm of dopamine levels in mouse striatum, which is abolished by surgical removal of the pineal gland, and then restored by daily MT injections (Khaldy et al., 2002).

The circadian system also provide a temporal framework for cognitive function in mammals (Gerstner et al., 2014). In particular, attention, memory, mood- and reward-related behaviors are regulated by the circadian clock in several species (Escribano & Diaz-Morales, 2014; Muller, Fritzsche, & Weinert, 2014; Sidor et al., 2015; Webb, Lehman, & Coolen, 2015). We recently reported that interval timing, a complex cognitive process, is also modulated by the circadian system (Agostino, do Nascimento, Bussi, Eguia, & Golombek, 2011). Time perception in the range from seconds to minutes, called interval timing, is crucial for multiple cognitive processes such as memory, learning and decision making (Buhusi & Meck, 2005; Lustig, Matell, & Meck, 2005). Experimental results indicate that certain brain areas including the prefrontal cortex, the basal ganglia, the striatum and its afferent projections from the substantia nigra pars compacta (SNPC) are necessary for interval timing (Buhusi & Meck, 2005). An adaptive dopaminergic function is also required; since DA availability alters the speed of interval timing processes (Coull, Cheng, & Meck, 2011; Meck, 2005; Meck et al., 2012). We have recently proposed dopamine signaling as a link between circadian and interval timing systems (Bussi, Levin, Golombek, & Agostino, 2014).

In the present work we studied the role of melatonin in the circadian regulation of interval timing. We propose that this hormone, as an output of the circadian system, is able to modulate temporal precision and dopamine levels in the striatum.

Materials and Methods

Animals

Wistar *(Rattus norvegicus albinus)* rats were purchased from commercial suppliers (Bioterio Central, Universidad Nacional de La Plata) and were maintained in a 12:12 hr light-dark cycle (LD, lights off at 0800 hr) with food and water *ad libitum* (except when noted) and room temperature set at 20 ± 2 ºC. Male adult (3-4 months old) animals were used throughout the experiments. When animals had to be handled in the dark, we used a dim red light source (<5 lux). The present experiments were approved by the Ethical Committee of the University of Quilmes (Buenos Aires, Argentina), and performed in strict accordance with NIH rules for animal care and maintenance.

Surgery

Rats (n = 16 for behavioral experiments and n = 20 for catecholamine quantification) were anesthetized intraperitoneally with ketamine (30 mg/kg) and xylazine (15 mg/kg) and placed in a stereotaxic frame for surgical removal of the pineal gland (n = 18) or sham surgery (n = 18). Pinealectomy (Pnx) was performed according to the method described in Hoffman and Reiter (1965), with slight modifications. Briefly, a hole was drilled in the skull, and the superior sagittal sinus was carefully deflected to minimize bleeding. In the PnX group, the pineal gland (which is located just below the posterior venous sinus confluence) was removed with a fine forceps. In sham-operated animals, the forceps were introduced in the vicinity of the pineal without injuring the gland or neighboring vascular and brain tissue. After surgery the rats were housed in individual cages in isolation cabinets until complete recovery. Three

weeks later, animals were progressively food-deprived during one week, after which the operant lever-press training was started.

Interval Timing Protocol

Apparatus. Experimental chambers (internal dimensions 30 x 22 x 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. Chambers were equipped with a 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 fixed interval (FI) and peak interval (PI) training, the stimulus was a 15-lux red light mounted at the center-top of the front wall.

Animals were trained in three consecutive phases: operant lever press training, fixed-interval training, and peak-interval training (Agostino, Cheng, Williams, West, & Meck, 2013; Bussi et al., 2014). In all segments of the experiment, sessions occurred once per day, 5 days per week (Monday through Friday).

Operant Lever-press Training. Rats 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. The lever was retracted after the 20th reinforcement, extended again after a variable delay, and then the cycle was repeated, in order to familiarize rats with the retraction and extension of the lever. After five days with this protocol, rats received a shorter continuous reinforcement training session. The session began with the lever extended. The lever was retracted every two reinforcements and then re-extended after a variable intertrial interval. The session ended when the animal earned 60 reinforcements or 1 hr had elapsed, whatever happened first. After another five days of this kind of sessions, rats underwent a fixed-interval (FI) training.

Fixed-interval (FI) Training. Lever presses were not reinforced until after a fixed interval had elapsed. Rats 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 and reached the criterion of 30 rewards in one session on the FI-24 s before moving them to the peak-interval training.

Peak-interval (PI) Training. After the FI training, rats received 78 daily 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 four 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.

Melatonin (MT) Administration

At the end of session 34, both sham and PnX rats were divided in two groups. From session 35 to 50 and 62 to 78 of PI training, half of the animals in sham and Pnx rats received melatonin (Sigma-Aldrich, St Louis, MO) administration at an expected dose of 1 mg/kg body weight/day. MT was dissolved in 0.1% ethanol aqueous solution, vehicle (VEH), and administered in drinking water. Although bottles were available through 24 hours, about 95% of their daily drinking occurs during the 12-hr dark period (Stephan & Zucker, 1972). The volume of water drunk by each rat was measured, the drug concentration was modified accordingly, and the actual doses were calculated on the basis of the volume consumed as previously published (Carpentieri, Pujolras, Chiesa, Noguera, & Cambras, 2006). The other half of the animals received VEH administration.

Jet-lag Simulation

For the *jet-lag* simulation experiment, rats were subjected to an abrupt 11-hr advance of the light/dark cycle. After this advance in the lighting schedule, peak interval training continued to occur at the

same clock hours than before the modification. Therefore, animals were trained and tested in the middle of the night before jet-lag simulation and in the middle of the day after advancing the light/dark cycle (see Figure 4A).

Two-bottle Preference Test

The sucrose preference test was carried out during two consecutive days. Two bottles were used, one filled with 5% sucrose solution and another with water. Placement of the bottles with sucrose and water was randomized across the tests. Liquid consumption was measured for the period of 1 hr by weighing pre-weighed bottles at the end of the test. Sucrose preference was measured by calculating the proportion of sucrose consumption out of total liquid consumption.

Catecholamine Quantification by Reverse-phase High-pressure Liquid Chromatography (HPLC-ED)

Animals were sacrificed by decapitation, and brains were quickly removed and kept at -80 °C. Dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) levels were determined from the supernatant of homogenized tissue from rat striatum. Samples were collected at ZT 18 (n = 5/group). Tissue was homogenized in 1 ml of 0.3 M perchloric acid, centrifuged for 15 min at 3000 g at 4 °C and then frozen at 80 °C. Samples were partially purified by batch alumina extraction, separated by HPLC-ED using a 4.6 x 250-mm Hypersil Gold C18 column (Thermo Fisher Scientific, Pittsburgh, PA, USA). Quantification was performed by current produced upon exposure of the column effluent to oxidizing and then reducing potentials in series using a triple-electrode system (Coulochem II; ESA, Bedford, MA, USA; (Eisenhofer et al., 1986). Catecholamine concentrations in each sample were corrected for recovery of an internal standard dihydroxybenzylamine. DA and DOPAC quantification was referred to total protein content. Proteins were measured by using the NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Data Analysis

Peak Curve Analysis. Data were used to estimate the peak time, peak rate, and precision of timing from the response functions for each animal. The number of responses (in 1 s bins) was averaged daily over trials, to obtain a mean response rate for each rat. Daily 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) to the data (Marquardt, 1963). The following generalized Gaussian + linear model was fit to the individual daily mean response rate functions:

$$R(t) = a \times \exp\text{¿¿} \qquad \text{Eq. 1}$$

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. Two-tailed t-tests were performed to compare peak location, peak height and peak width between groups.

Given the observation that separate thresholds may be used to start and stop responding around a criterion time, and that differences in these parameters may reflect distinct mechanisms, data were also used to calculate these response thresholds (Cheng, Etchegaray, & Meck, 2007; Church, Meck, & Gibbon, 1994). From the average response rates, start and stop response thresholds (S1 and S2 rate indexes, respectively) were calculated as previously described (Bussi et al., 2014). Briefly, the S1 rate index for the PI-24 s training was defined by the response rate occurring during the 3 s period just prior to the time of reinforcement (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 time of reinforcement (i.e., seconds 24–26) divided by the overall response rate during the last 72 s of the trial (i.e., seconds 24–96). Higher values of S1 and S2 rate indexes indicate sharper FI or PI timing functions and better duration discrimination. During FI training, only S1 rate index was calculated. To analyze the S1 and S2 rate indexes across sessions, a mixed-design (two-way repeated measures) ANOVA

was performed, with sham or PnX conditions as the between-subjects factor. When assumptions for parametric ANOVA were not met, the Friedman's two-way analysis of variance was used.

Single Trial Analysis

Analysis of responses in individual peak trials was performed as previously reported (Balci et al., 2010; Church et al., 1994; Gallistel, King, & McDonald, 2004; Matell, Bateson, & Meck, 2006). The transition into (start time) and out of (stop time) the high rate was defined by the point at which the data first exceeded or fell below, respectively, 70% of the maximum response rate. Peak time was defined as the middle point between start and stop times. Spread was defined as the difference between start and stop times. Single-trial analysis was performed only for trials in which rats exhibited *good timing* (about 65%), that is, response onset prior to the criterion time, response offset following criterion time, and a low-high-low step function (Cheng et al., 2007; Church et al., 1994; Taylor, Horvitz, & Balsam, 2007). Single trial parameters were compared by using one-way ANOVA or two-tailed *t*-tests. When assumptions for parametric analysis were not met, nonparametric Mann-Whitney *U* test was used.

For the two-bottle preference test, differences between groups were assessed by Student's *t*-test. Dopamine levels in sham and PnX rats were analyzed by using two-way ANOVA. Statistical analyses were performed using Graphpad Prism (GraphPad Software Inc., CA, USA), excepting the Friedman's two-way repeated measures ANOVA that was performed using MATLAB software. In all cases, the alpha level was set at $p < 0.05$.

Results

1. Effect of melatonin depletion on interval timing

In order to elucidate the role of MT on interval timing, rats were MT-depleted through removal of the pineal gland. Since we used 5% sucrose in water as reward, a two-bottle choice test was performed to determine differences in sucrose preference between groups. Both pinealectomized (PnX) and controls (sham) rats had similar preference for sucrose intake, $t(14) = 0.31$, $p = 0.76$ (two-tailed *t*-test, $n = 8$/group). Four weeks after surgery, sham and PnX rats were trained by using the PI schedule. During operant lever-press training, rats received ten continuous-reinforcement schedule sessions to learn the association between lever press and reward delivery. There were no differences in the speed (number of sessions) with which this response was acquired, $F(4, 60) = 0.36$, $p = 0.55$ (mixed-design ANOVA) nor the number of total lever presses, $t(14) = 1.03$, $p = 0.32$ (two-tailed *t*-test, $n = 8$/group). Acquisition of temporal control during fixed interval (FI) training was evaluated using the mean S1 rate index in both groups for the 24 s target duration. Although the S1 rate index was slightly higher for sham rats, this difference did not reach significant levels, $F(7, 105) = 7.41$, $p < 0.001$ for sessions, $F(1, 63) = 3.98$, $p = 0.06$ for groups (mixed-design ANOVA).

The mean proportion of maximum response rate plotted as a function of time for the last session block (session 29-32) of peak interval (PI) training is shown in Figure 1. Both groups acquired temporal control of their responses as a function of time in probe trials, producing a Gaussian-shaped response function centered at the expected time of reinforcement (Figures 1A and B for sham and PnX rats, respectively). Figures 1C to E show the main parameters from the Gaussian function: peak location, peak amplitude, and peak width. The PnX rats showed diminished amplitude and greater dispersion compared to the control rats, reflected on lower values in peak height and higher values

in peak width (Figures 1D and E, respectively; $t(14) = 0.17$, $p = 0.87$ for peak location; $t(14) = 2.46$, $p = 0.03$ for peak height, and $t(14) = 2.64$, $p = 0.02$ for peak width; two-tailed t-test, $n = 8$/group).

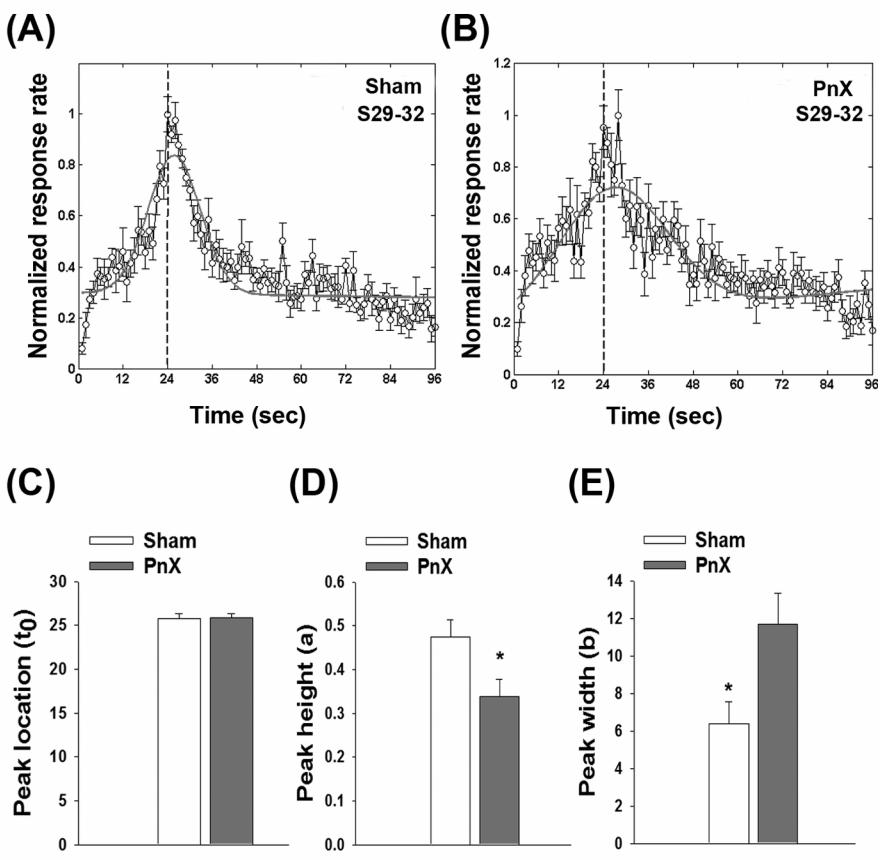

Figure 1. Effect of melatonin depletion on interval timing. Normalized response rate of PI trials as a function of time in the trial in either sham (A) or PnX (B) rats during the last 4-session block (sessions 29-32) of PI training. Rats were trained and tested during the middle of the night at ZT 15-17. Dashed line indicates target time (24 s). The solid curves indicate best-fit Gaussian function to the average response curves. (C) to (E) show the mean best-fit Gaussian parameter estimates. (C) Peak location, (D) peak height, and (E) peak width. *$p <$ 0.05 (two-tailed t test). Data are shown as M + SEM (n = 8/group).

The mean S1 and S2 rate indexes were used to evaluate learning of the start and stop responding, respectively, as previously reported (Agostino et al., 2011; Cheng et al., 2007). Figure 2 shows the mean S1 (A) and S2 (B) rate indexes from sham and PnX rats. Both groups showed an increase in these indexes across sessions, suggesting that sham and PnX rats were able to learn to start and stop responding around the target interval. However, sham animals reached higher values for those indexes, suggesting a better performance compared to PnX in the acquisition of these behaviors (mean S1 rate index: $F(7, 105) = 10.33$, $p < 0.001$ for sessions, $F(1, 63) = 26.46$, $p < 0.001$ for groups; mean S2 rate index: $F(7, 105) = 27.70$, $p < 0.001$ for sessions, $F(1, 63) = 54.74$, $p < 0.001$ for groups; mixed-design ANOVA). Single trial analysis was performed with data collected from sessions 13-16 (middle block of PI training) and 29-32 (last session block; Figure 2C). In the middle block of PI training, differences in start and peak time values were found between groups ($t(14) = 3.31$, $p = 0.01$ for start time; $t(14) = 1.65$,

p = 0.12 for stop time, $t(14)$ = 3.94, p = 0.002 for peak time, and $t(14)$ = 1.03, p = 0.32 for spread, two-tailed t-test). PnX rats showed earlier responses compared to sham, producing lower values of start time and peak time. No differences were found during the last block of sessions ($t(14)$ = 0.38, p = 0.71 for start time; $t(14)$ = 0.72, p = 0.48 for stop time, $t(14)$ = 0.74, p = 0.47 for peak time, and $t(14)$ = 0.32, p = 0.75 for spread, two-tailed t-test). These results indicate that PnX rats needed more training than sham rats for learning the timing task. However, according to single trial analysis, after 32 sessions both groups exhibit similar performances.

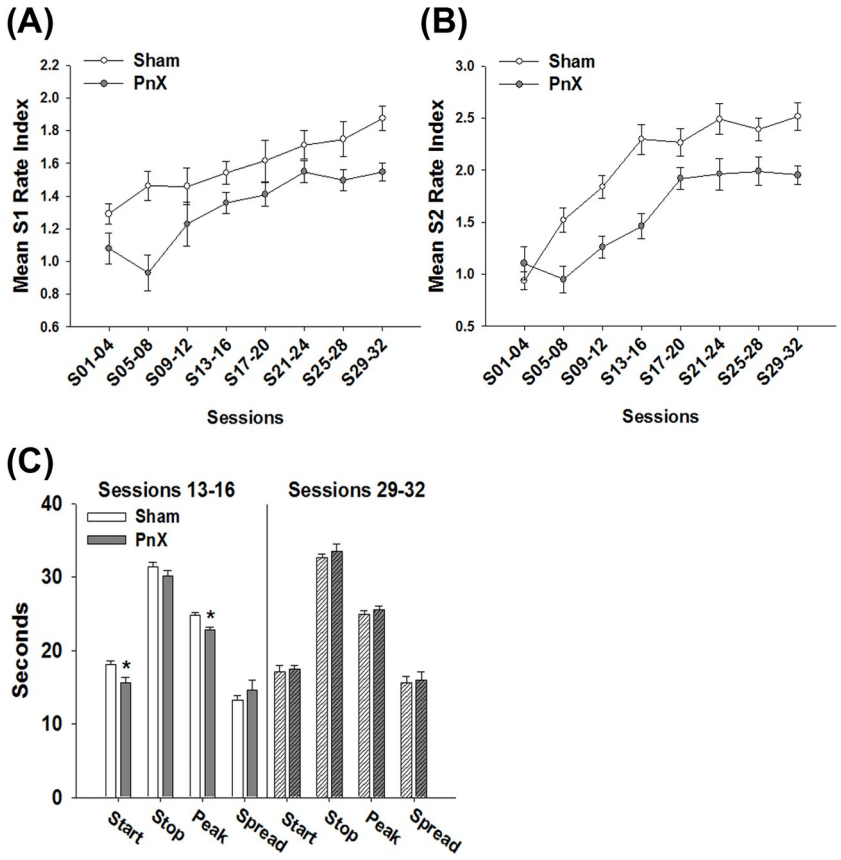

Figure 2. Quantification of the effect of melatonin depletion on interval timing. Mean S1 (A) or S2 (B) rate indices across sessions for either sham (white circles) or PnX (grey circles) rats. Sessions are plotted as blocks of 4 sessions each. $p < 0.001$ for sessions, $p < 0.001$ for groups for both S1 and S2 rate indices, mixed-design (two-way repeated measures) ANOVA. (C) Mean start time, stop time, peak time and spread from single-trial analyses from the fourth block (B4, sessions 13-16) and the last block (B8, sessions 29-32) of peak-interval training. *$p < 0.05$ (two-tailed t-test). Data are shown as $M \pm SEM$ (n = 8/group).

2. Melatonin restores timing precision in PnX rats.

In order to test if MT could restore the precision in estimating the given interval, this hormone was administered in the drinking water starting at session 35 of PI training. Both sham and PnX rats received either MT or vehicle (0.1% ethanol in water) for the next 16 PI training sessions (sessions 35 to 50). Figure 3 shows the mean proportion of maximum response rate plotted as a function of time during MT administration for the last session block (sessions 47-50) of PI training. There were no

significant differences between MT-treated groups in the representative parameters of the Gaussian function (U = 4.00, p = 0.34 for peak location; U=6.00, p = 0.69 for peak height, and U = 5.00, p = 0.49 for peak width, two-tailed non-parametric Mann-Whitney U test for the last session block of PI training, sessions 47-50, n = 4/group).

Figures 3C and D show the mean S1 and S2 rate indexes, respectively, during melatonin administration. For both indexes, the PnX group exhibited an improvement in the first 4-session block (sessions 35-38), reaching similar levels than control rats. Moreover, there were no differences between MT-treated groups during the next 12 sessions with MT administration (mean S1 rate index: $F(3, 21) = 4.19$, $p = 0.01$ for sessions, $F(1, 15) = 0.20$, $p = 0.65$ for groups; mean S2 rate index: $F(3, 21) = 3.26$, $p = 0.03$ for sessions, $F(1, 15) = 0.52$, $p = 0.50$ for groups, Friedman's two-way repeated-measures ANOVA). Importantly, PnX + MT rats showed significant differences in their rate indexes when compared to the PnX + VEH group (mean S1 rate index: $F(1, 15) = 124.8$, $p < 0.0001$; mean S2 rate index: $F(1, 15) = 91.62$, $p < 0.001$, Friedman's two-way repeated-measures ANOVA). Thus, PnX rats without MT administration did not present improvement in the timing task.

Single trial analysis was performed for the first block (sessions 35-39, Figure 3E) and the last block (sessions 47-50, Figure 3F) of PI training during MT administration. There were no significant differences between MT-treated sham or PnX rats in single trial parameters (sessions 35-38: $F(2, 9) = 0.53$, $p = 0.61$ for start time; $F(2, 9) = 0.06$, $p = 0.94$ for stop time, $F(2, 9) = 0.11$, $p = 0.90$ for peak time, and $F(2, 9) = 0.22$, $p = 0.81$ for spread; sessions 47-50: $F(2, 9) = 11.17$, $p = 0.004$ for start time; $F(2, 9) = 1.16$, $p = 0.36$ for stop time, $F(2, 9) = 2.57$, $p = 0.13$ for peak time, and $F(2, 9) = 4.80$, $p = 0.04$ for spread, one-way ANOVA). Tukey's multiple comparison test indicated significant differences for PnX + VEH versus MT-treated groups for start time and spread at sessions 47-50 (Figure 3E). There was no effect of MT treatment in the sham group (sham + MT vs. sham + VEH, data not shown). These results indicate that melatonin was able to restore timing precision in PnX rats.

3. The effect of melatonin is time-specific.

Our previous results indicated both daily and circadian differences in interval timing accuracy and precision in mice, with better performance on the timing task (i.e., peak location closer to the target time, higher peak amplitude and reduced peak width) in the nocturnal phase of the light/dark (LD) cycle (Agostino et al., 2011). In order to test if MT administration could also improve interval timing during the daytime, sham and Pnx rats were subjected to an advance of the LD cycle (jet-lag simulation) at the end of the session 50 of PI training. After an abrupt change of the LD cycle, circadian rhythms gradually adapt to the new environmental conditions. In this particular situation, resynchronization to the new cycle usually takes 8-10 days in rodents (Agostino, Plano, & Golombek, 2007; Kiessling, Eichele, & Oster, 2010). After jet-lag simulation, rats received 12 additional sessions of PI training, receiving training during the daytime of the LD cycle (Figure 4A). As previously observed in mice (Agostino et al., 2011), interval timing was significantly affected by changing the light schedule. There was a dramatic decrease in the mean S1 rate index for both groups after jet-lag simulation (Figure 4B, $F(1, 7) = 6.44$, $p = 0.01$ for sessions, $F(1, 7) = 1.05$, $p = 0.30$ for

groups, Friedman's two-way repeated-measures ANOVA comparing the session block immediately after jet-lag [sessions 51-54] with the session block before jet-lag [sessions 47-50]). This effect was also observed in PnX rats for the mean S2 rate index (Figure 4C), with sham rats affected less by the abrupt advance of the LD cycle ($F(1, 7) = 20.02$, $p = 0.003$ for sessions, $F(1, 7) = 4.69$, $p = 0.03$ for groups, Friedman's two-way repeated-measures ANOVA comparing session blocks before and after jet-lag). For clarification, only previously MT-treated groups are shown. Single

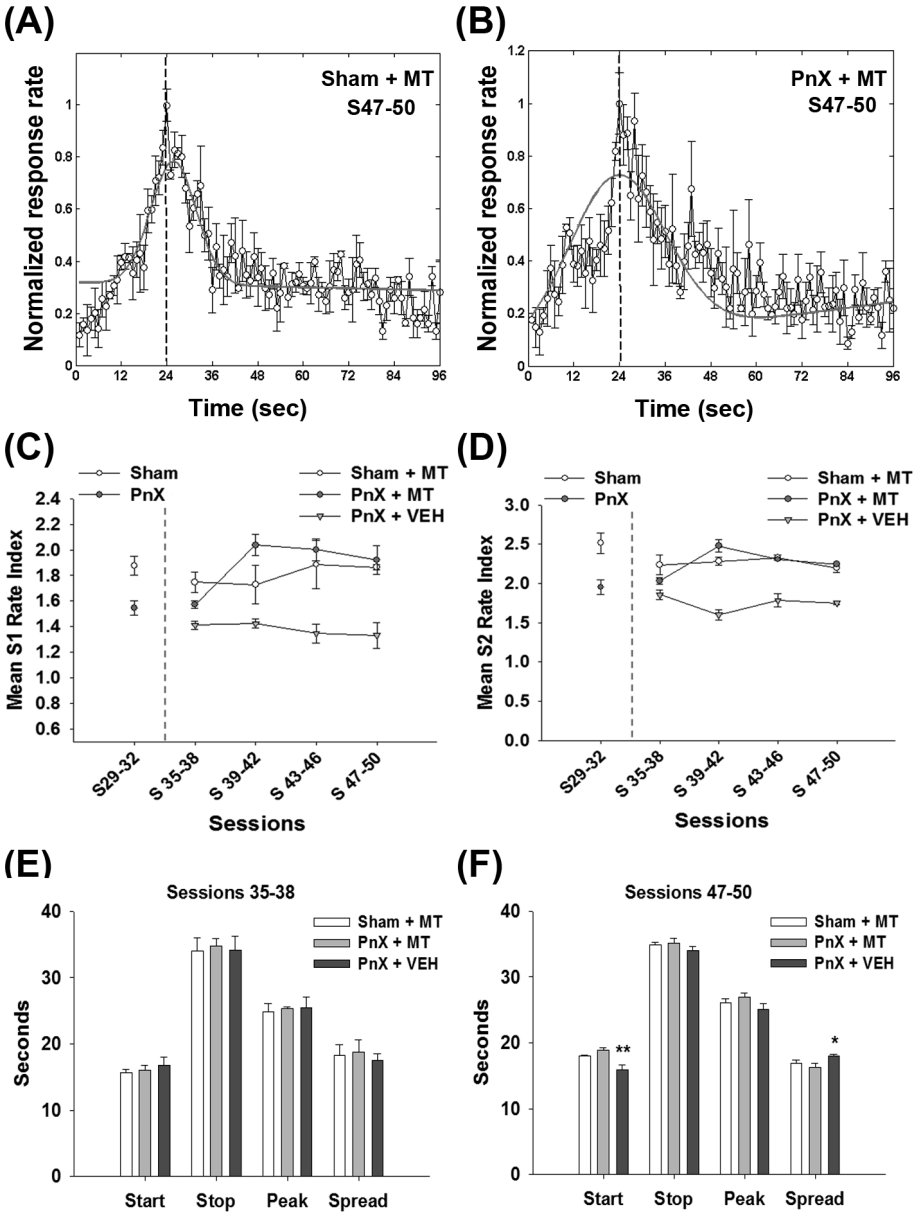

Figure 3. Restoration of interval timing in PnX rats by melatonin administration. Normalized response rate of PI trials as a function of time in the trial in either sham (A) or PnX (B) rats during the last 4-session block (sessions 47-50) of PI training with melatonin administration in drinking water. Rats were trained and tested during the middle of the night at ZT 15-17. Dashed line indicates target time (24 s). Straight line indicates best fit to a Gaussian function. (C) and (D) show the mean S1 (C) or S2 (D) rate indices across sessions for either sham (white circles) or PnX (grey circles) rats. Sessions are plotted as blocks of 4 sessions each. For comparison, previous pre-melatonin 4-session block is shown (sessions 29-32) on the left side. S1 rate index: $p < 0.01$ for sessions, $p > 0.05$ for groups; S2 rate index: $p < 0.05$ for sessions, $p > 0.05$ for groups, Friedman's two-way repeated measures ANOVA. (E) and (F) Mean start time, stop time, peak time and spread from single-trial analyses from the first block (sessions 35-39) and the last block (sessions 47-50) of peak-interval training during melatonin administration. *$p < 0.05$ (one-way ANOVA for each parameter). Data are shown

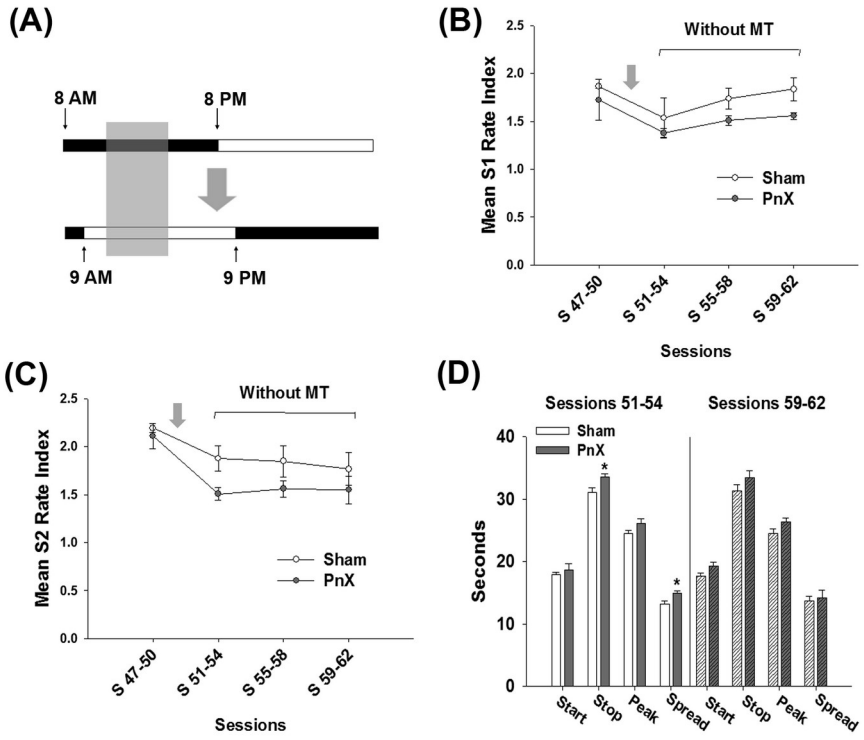

Figure 4. Circadian desynchronization affects interval timing in rats. (A) Schematic illustration of the protocol used for circadian desynchronization (jet-lag simulation). An eleven-hour phase advance was conducted starting by the light phase. The grey bar indicates the time of training and testing, which was maintained through change of the light/dark cycle. (B) and (C) show the mean S1 (B) or S2 (C) rate indices across sessions for either sham (white circles) or PnX (grey circles) rats. Sessions are plotted as blocks of 4 sessions each. Arrow indicates the change of the light/dark cycle. S1 rate index: $p < 0.05$ for sessions, $p > 0.05$ for groups; S2 rate index: $p < 0.001$ for sessions, $p < 0.05$ for groups, Friedman's two-way repeated measures ANOVA. (D) Mean start time, stop time, peak time and spread

trial analysis revealed that there was an increase in stop time and spread in PnX rats in the block of sessions immediately after jet-lag simulation (Figure 4D, sessions 51-54: $U = 6.00$, $p = 0.69$ for start time; $U = 0.00$, $p = 0.029$ for stop time, $U = 3.50$, $p = 0.25$ for peak time, and $U = 0.00$, $p = 0.028$ for spread; sessions 59-62: $U = 2.00$, $p = 0.11$ for start time; $U = 5.00$, $p = 0.49$ for stop time, $U = 3.00$, $p = 0.20$ for peak time, and $U = 7.00$, $p = 0.89$ for spread, two-tailed non-parametric Mann-Whitney U test, $n = 4$/group), indicating a major negative effect of changing the LD cycle in rats without endogenous MT.

After animals adapted to the new LD cycle, MT was administered again in drinking water. Then, rats were subjected to 16 additional training sessions during the daytime. PnX and sham rats showed no significant differences in the mean S1 rate index ($F(3, 21) = 0.80$, $p = 0.85$ for sessions, $F(1, 15) = 4.30$, $p = 0.11$ for groups, two-way Friedman's repeated-measures ANOVA, Figure 5A). However, the mean S2 rate index was affected in the sham group along sessions, whereas it did not show significant changes in the PnX group ($F(3, 21) = 0.85$, $p = 0.50$ for sessions, $F(1, 15) = 31.92$, $p = 0.005$ for groups, two-way Friedman's repeated-measures ANOVA, Figure 5B). There were no modifications in the S1 or S2 rate indexes in VEH-treated animals

(data not shown). Moreover, single trial analysis was performed for the last block (sessions 75-78, Figure 5C), with no significant differences between groups (U = 5.00, p = 0.49 for start time; U = 6.00, p = 0.69 for stop time, U = 6.00, p = 0.69 for peak time, and U = 6.5, p = 0.77 for spread, two-tailed non-parametric Mann-Whitney U test, n = 4/group). These results suggest that MT administration did not improve timing performance during the daytime.

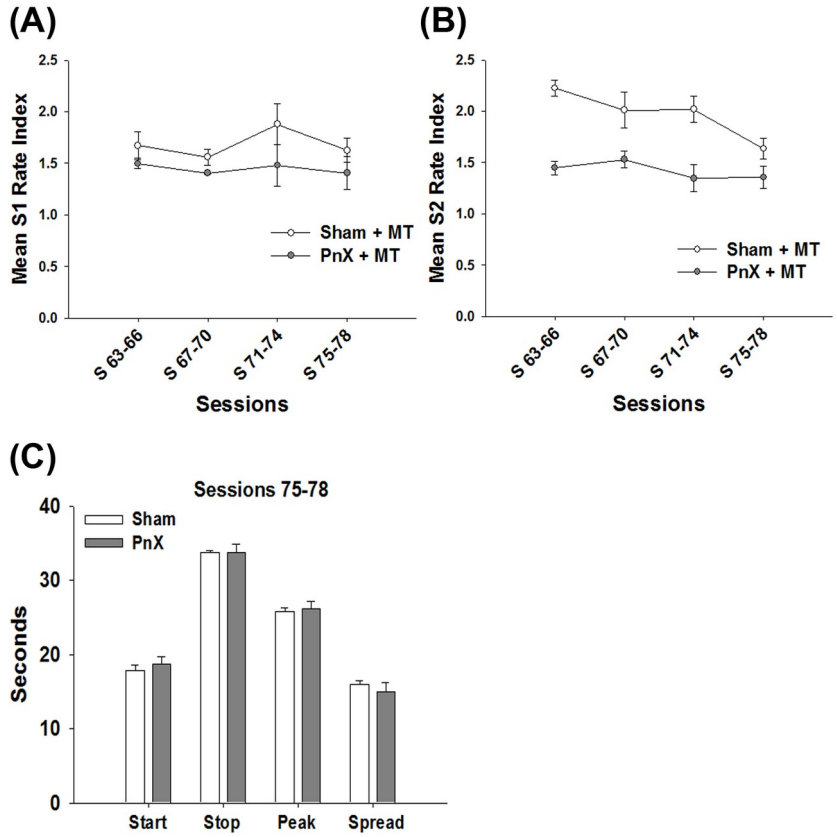

Figure 5. Melatonin administration and interval timing during the day. The mean S1 (A) or S2 (B) rate indices across sessions are shown for either sham (white circles) or PnX (grey circles) rats. Sessions are plotted as blocks of 4 sessions each. Mean S1 rate index: $p > 0.05$ for sessions, $p > 0.05$ for groups; mean S2 rate index: $p > 0.05$ for sessions $p < 0.01$ for groups, Friedman's two-way repeated measures ANOVA. (C) Mean start time, stop time, peak time and spread from single-trial analyses from the last block (sessions 75-79). Data are shown as

4. Melatonin affects dopamine levels in the striatum.

An optimal dopaminergic function is required for interval timing processes (Meck, 2005). Our previous results indicated that striatal dopamine availability correlated with interval timing accuracy and precision in mice (Bussi et al., 2014). Moreover, previous studies demonstrated that there was a negative correlation between MT and dopaminergic activity in several brain structures (Dominguez-Lopez, Howell, Lopez-Canul, Leyton, & Gobbi, 2014; Zisapel, 2001). In order to test if the restorative effect of MT on timing performance in PnX rats during the nighttime was related to the changes in striatal dopamine levels, the amount of dopamine was measured by HPLC-ED in sham

and PnX rats with either MT or VEH administration. Figure 6 shows that pinealectomy increased the amount of striatal dopamine in rats, and this increase was reverted by exogenous MT administration. There were no significant changes in DOPAC levels ($F(1, 9)=3.39$, $p = 0.084$ for treatment, $F(1, 9) = 0.004$, $p = 0.998$ for groups, two-way ANOVA). Moreover, MT administration did not modify striatal dopamine levels in control (sham) rats ($F(1, 9) = 7.53$, $p = 0.01$ for treatment, $F(1, 9) = 8.70$, $p = 0.009$ for groups, two-way ANOVA).

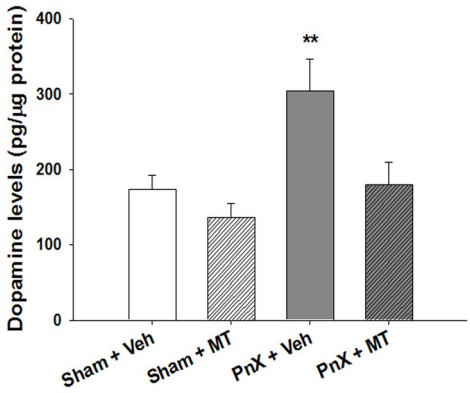

Figure 6. Striatal dopamine levels. DA was measured by HPLC-ED. Samples were taken at ZT 18 from sham (white bars) and PnX (grey bars) rats with melatonin (striped bars) or vehicle (clear bars) administration. *$p < 0.05$ for groups (sham vs. PnX); **$p < 0.01$ for treatment (MT vs. VEH), two-way ANOVA. **$p < 0.01$ for vehicle administration in the PnX rats vs. all others groups, Bonferroni's post hoc test. Data are expressed as $M \pm SEM$ ($n = 5$/group).

Discussion

The circadian system regulates several physiological and behavioral functions with a period near to 24 hours. The cognitive performance indeed depends on the time of day but there is little information about the specific mechanism that underlays this regulation (Escribano & Diaz-Morales, 2014; Muller et al., 2014; Sidor et al., 2015; Webb et al., 2015). Interval timing, a complex cognitive process, is also modulated by the circadian system. We have previously proposed that dopaminergic signaling might serve as a possible link between both temporal systems (Bussi et al., 2014). Dopamine is one of the most important neurotransmitters influencing timing behavior, and several components of dopaminergic signaling have their expression subjected to circadian control (Golombek, Bussi, & Agostino, 2014).

Melatonin, a circadian hormone, acts as a *time-giver* to indicate the time of environmental darkness, in both diurnal and nocturnal animals. This hormone is involved in several physiological processes such as the regulation of seasonal reproduction, body weight and energy balance (Barrenetxe, Delagrange, & Martinez, 2004). Several studies showed significant effects of MT depletion on different tissues. For example, pinealectomy induced morphological changes and neurogenesis impairment in the hippocampus (Fisher & Sugden, 2010; Kus et al., 2013; Rennie, De Butte, & Pappas, 2009). In addition, the absence of MT caused the loss of a daily rhythm in striatal dopamine levels in mice (Khaldy et al., 2002). Melatonin depletion also

caused behavioral anomalies such as an increase in anxiety-like and depressive-like behavior (Zahra, Siham, Abdelhalim, Aboubakr, & Ali, 2012).

In the present work we show that pinealectomy impairs interval timing accuracy (by shifting peak time) and precision (by increasing peak width) in rats (Figures 1 and 2). The analysis of the average response curve revealed a more dispersed response in PnX rats. There were significant differences in the Gaussian parameters after 32 sessions of PI training, with higher values in peak width and lower values in peak height in PnX rats compared to control (sham) animals. Moreover, single trial analysis indicated significant differences in start time and peak time in the middle point of the training schedule (sessions 13-16), with earlier start and peak times in the PnX group. These differences were not maintained in the last block of PI training (sessions 29-32, Figure 2), indicating that with extensive training both groups reach similar performances. In single trial analysis, the start time has been reported to be more sensitive to the motivational state (Balci, 2014), and earlier start times correlate with higher tonic dopamine levels (Balci et al., 2010). Indeed, several studies showed that an increase in dopamine levels by pharmacological and genetic approaches produces a leftward shift in peak time (Buhusi & Meck, 2005; Gooch, Wiener, Portugal, & Matell, 2007; Meck, 1996; Taylor et al., 2007). This observation has been attributed to an increase in internal clock speed resulting from elevated dopamine levels (Buhusi & Meck, 2005; Meck, 1996). However, some studies failed to replicate these results (Balci et al., 2008; Chiang et al., 2000). This discrepancy could be consequence of changes in important variables, such as the data analysis or the number of sessions before testing.

On the other hand, an increase in reward magnitude appears to have the same effect that an increase in dopamine levels, that is, earlier start times and a left shift in peak times (Galtress, Garcia, & Kirkpatrick, 2012; Ludvig, Conover, & Shizgal, 2007). Our results indicate that MT depletion transiently affects the Start and Peak times during the PI procedure. This effect was transitory, since PnX rats improved their performance across sessions, showing no significant differences in single trial analysis at the last block of training. However, analysis of the average response curve indicated that the PnX group, although improved across sessions, did not reach same levels as the control rats (Figure 2). The differences found between average and single trial results have been described by previous studies (Balci, 2014; Taylor et al., 2007). Generally, individual performances are better characterized by using single trial analysis, because in the average data the peak location is vulnerable to changes in start or stop times. Hence, a leftward shift in peak location could be produced by earlier start times without affecting stop times. These details are better described by single trial analysis. The improvement observed in PnX rats could be explained by the fact that the MT daily rhythm is not the only efferent signal from the circadian clock. Indeed, for a given function, even without MT (e.g., after pinealectomy), circadian modulation can be executed via other, nervous or hormonal, clock outputs (Pevet & Challet, 2011).

In addition, here we demonstrate that exogenous MT administration to PnX rats causes an improvement in timing behavior, resulting in Gaussian parameter values that are similar to the control rats (Figure 3). Analysis of the average response curve revealed that there were no significant differences in the Gaussian parameters between MT-treated groups, indicating that both groups had similar performances in the timing task (Figures 3A and B). Moreover, this improvement was reflected by an increase in the

mean S1 and S2 rate indexes in the PnX + MT group. In contrast, PnX rats without MT (PnX + VEH group) did not exhibit any progress or enhancement in the timing task (Figures 3C and D). Single trial analysis also revealed no significant differences between MT-treated groups (Figures 3E and F). However, there was a significant difference in start time and spread between MT-treated rats and VEH administration in the last session block of training under MT administration (sessions 47-50). MT caused an increase (rightward shift) in start time in PnX rats, whereas PnX + VEH animals exhibited earlier start times (Figure 3F). Also, spread was significantly higher in the PnX + VEH group. These results indicate that exogenous MT application improves interval timing in PnX rats. In addition, there was no effect of MT administration on sham rats, suggesting that the high enough levels of MT in these control animals might have masked the effect of exogenous MT.

Environmental disturbances of the circadian clock, such as shiftwork or jet-lag schedules, compromise sleep, alertness and problem solving in humans (Kyriacou & Hastings, 2010; Wright, Lowry, & Lebourgeois, 2012). There is clear evidence that chronic phase shifts of the light/dark cycle interfere with memory in rats and mice (Devan et al., 2001; Fekete, van Ree, Niesink, & de Wied, 1985; Loh et al., 2010). After an abrupt change of the light/dark cycle, circadian rhythms gradually adapt to the new environmental conditions. The same adaptation was observed for cognitive performance in mice and humans (Agostino et al., 2011; Soshi et al., 2010). Consistent with these previous studies, we demonstrated that rats suffer a transient impairment in interval timing after jet-lag simulation (Figure 4). Average analysis showed that both sham and PnX rats exhibited a transient decrement in their mean S1 rate index. Moreover, the mean S2 parameter decreased in the PnX rats during the sessions following jet-lag simulation. Single trial analysis indicated that only the PnX rats showed an increase in stop time and spread after jet-lag simulation, indicating a decrease in timing precision. It has been reported that acquisition of stop time is related to protein synthesis in the ventral striatum (VS), and that the VS may modulate changes in the reward value associated with feedback at the temporal criterion (Macdonald, Cheng, & Meck, 2012), Thus, jet-lag simulation might generate a misalignment of reward processing, which may be related to the decreased precision of timing. This is in accordance with a recent study which shows that reward-related neurophysiology and behavior are under circadian modulation (Webb et al., 2015). In contrast, sham rats did not exhibit significant changes in these parameters, suggesting that endogenous MT plays a protective role inhibiting the misalignment caused by a jet-lag simulation.

Although the suprachiasmatic nuclei and some clock proteins appear to be unnecessary for interval timing (Cordes & Gallistel, 2008; Lewis, Miall, Daan, & Kacelnik, 2003; Papachristos, Jacobs, & Elgersma, 2011), time-of-day effects have been observed for the timing of auditory and visual signals in the seconds-to-minutes range (Kuriyama et al., 2005; Lustig & Meck, 2001; Meck, 1991; Pati & Gupta, 1994). Indeed, our results indicate that time perception exhibits circadian variations in rats and mice, with better performance during the night. During the day, in which rodents are not active, their performance in several cognitive tasks is lower (Agostino et al., 2011; Gritton, Kantorowski, Sarter, & Lee, 2012; Shurtleff, Raslear, & Simmons, 1990). In accordance with the results of these earlier studies, we found that interval timing in rats is less accurate during the day. Whereas 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 variations.

Previous research revealed that MT administration enhances cognitive performance under several conditions associated with cognitive impairment, such as psychiatric diseases and aging (Esteban et al., 2010; Liu et al., 2013; Peck, LeGoff, Ahmed, & Goebert, 2004). Our results indicate that MT improvement on interval timing is time-specific, since MT administration did not improve the performance of the sham nor PnX rats on the timing task when they were tested during the daytime (Figure 5). This result suggests that training during the daytime is relatively independent from MT. In this way, MT may be able to modulate timing behavior only during the nighttime, when the system is sensitive to this hormone.

Recent studies reported that striatal dopamine levels show a daily oscillation, with higher levels during the night in mice and rats (Bussi et al., 2014; Ferris et al., 2014). Moreover, removal of the pineal gland in mice caused the loss of striatal dopaminergic daily rhythm, which was restored by exogenous MT administration (Khaldy et al., 2002). Our data indicate that MT depletion causes a significant increase in striatal dopamine availability, and that MT administration in drinking water reversed this increase. This result is in accordance with previous research which showed that MT levels have a negative correlation with dopamine levels in several tissues and brain structures (Doyle et al., 2002; Zisapel, 2001). Moreover, MT administration did not modify dopamine levels in the sham rats, which is in agreement with previous studies that report a weak effect of exogenous MT when the endogenous hormone is present (Pevet & Challet, 2011). Furthermore, our results are in accordance with the report that an increase in dopamine availability decreased start time threshold and induced a leftward shift in peak time, which was related to the motivational state of the animal (Balci, 2014). In the present work, the PnX rats without MT administration exhibited earlier start times compared to the MT-treated groups (Figure 3F). This is also in accordance with our previous research in which we demonstrated that the circadian system is able to modulate interval timing by acting on the dopaminergic function in timing related brain structures (Bussi et al., 2014).

To summarize, in the present work we included the hormone melatonin as a circadian output that can mediate the interaction between circadian and interval timing, two time-processing systems that have been typically treated as independent. Although we acknowledge a number of limitations in the present study, such as small sample size or the disadvantage of null hypothesis significance testing to some data analysis, which could be better addressed with the use of Bayesian methods, our data indicate that MT-depleted rats showed an impairment in interval timing which was restored by exogenous MT administration. Thus, MT may act as a circadian output in brain structures related to reward processing and interval timing, such as the striatum and substantia nigra, both of them expressing functional MT receptors. In these structures, the pineal hormone may be a synchronizer, controlling the expression of circadian clock genes. The products of these genes, the circadian clock proteins, act as transcription factors regulating the expression of several other genes, some of which are the components of dopaminergic signaling (Golombek et al., 2014). Further research is needed to describe in detail the exact neurochemical and molecular connections between circadian and interval timers.

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