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Involvement of dopamine signaling in the circadian modulation of interval timing

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Abstract

Duration discrimination within the seconds-to-minutes range, known as interval timing, involves the interaction of cortico-striatal circuits via dopaminergic–glutamatergic pathways. Besides interval timing, most (if not all) organisms exhibit circadian rhythms in physiological, metabolic and behavioral functions with periods close to 24 h. We have previously reported that both circadian disruption and desynchronization impaired interval timing in mice. In this work we studied the involvement of dopamine (DA) signaling in the interaction between circadian and interval timing. We report that daily injections of levodopa improved timing performance in the peak-interval procedure in C57BL/6 mice with circadian disruptions, suggesting that a daily increase of DA is necessary for an accurate performance in the timing task. Moreover, striatal DA levels measured by reverse-phase high-pressure liquid chromatography indicated a daily rhythm under light/dark conditions. This daily variation was affected by inducing circadian disruption under constant light (LL). We also demonstrated a daily oscillation in tyrosine hydroxylase levels, DA turnover (3,4-dihydroxyphenylacetic acid/DA levels), and both mRNA and protein levels of the circadian component Period2 (Per2) in the striatum and substantia nigra, two brain areas relevant for interval timing. None of these oscillations persisted under LL conditions. We suggest that the lack of DA rhythmicity in the striatum under LL – probably regulated by Per2 – could be responsible for impaired performance in the timing task. Our findings add further support to the notion that circadian and interval timing share some common processes, interacting at the level of the dopaminergic system.

Introduction

Living organisms have developed the ability to fit behaviors to regular schedules around different timescales as a strategy to adapt to the environment. 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 *et al.*, 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, are necessary for interval timing (Buhusi & Meck, 2005). An optimal dopaminergic function is also required, as dopamine (DA) availability alters the speed of interval timing processes (Meck *et al.*, 2008, 2012; Coull *et al.*, 2011). In addition, recent studies have demonstrated the importance of specific elements in DA transmission on interval timing. In particular, a transient overexpression of striatal D2 receptors leads to an impairment in timing precision and accuracy (Drew *et al.*, 2007) probably by means of a motivational effect (Ward *et al.*, 2009). Moreover, the disruption of prefrontal D1 receptor signaling impairs temporal control in rats (Narayanan *et al.*, 2012). Furthermore, the downregulation of DA transporter (DAT) produces earlier responses in mice under the peak-interval (PI) protocol (Balci *et al.*, 2010), while DAT knockout mice show total loss of temporal control (Meck *et al.*, 2012). In humans, some DA-related gene polymorphisms (such as DRD2/ANKK1-Taq1a, COMT Val158Met and DAT 3′ VNTR) have been associated to timing functioning (Portnova *et al.*, 2007; Wiener *et al.*, 2011; Balci *et al.*, 2013).

On a much larger scale, circadian rhythms control physiological, behavioral and metabolic functions with periods close to 24 h (Dunlap *et al.*, 2004; Bass & Takahashi, 2010). At the molecular level, these rhythms emerge from transcriptional–translational feedback loops of core clock genes (Lowrey & Takahashi, 2004). In mammals, the circadian system is mainly synchronized by the light/dark (LD) cycle (Golombek & Rosenstein, 2010).

Previous studies have demonstrated a link between the circadian system and dopaminergic transmission (reviewed in Agostino *et al.*, 2011b; Golombek *et al.*, 2014). DA and its related metabolites exhibit daily fluctuations in different brain regions (Kafka *et al.*, 1986). Furthermore, some elements of the dopaminergic system, such as DAT and tyrosine hydroxylase (TH; the rate-limiting enzyme in DA synthesis) exhibit a diurnal rhythm in the medial prefrontal cortex, nucleus accumbens and striatum (Sleipness *et al.*, 2007).

In the present work, we studied the involvement of dopaminergic signaling in the circadian modulation of interval timing using both

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behavioral and molecular approaches. The PI procedure (Catania, 1970; Roberts, 1981; Church *et al.*, 1994; Paule *et al.*, 1999) was used to assess the effect of DA levels in time estimation performance in mice. In addition, we assessed rhythmicity in the dopaminergic system in correlation with behavioral performance.

Materials and methods

Animals

Across experiments, a total of 152 experimentally naïve mice (C57BL/6) were used. Animals were purchased from commercial suppliers (Bioterio Central, Universidad Nacional de La Plata), and were maintained in a 12 : 12 h LD cycle (lights ON at 08:00 h) with food and water *ad libitum* (except when noted) and room temperature set at 20 ± 2 °C. 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.

Locomotor activity recording

Animals were transferred to individual cages equipped with a running wheel (17 cm diameter) and with light intensity averaging 200 lux 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 : 12 h LD cycle. For constant light (LL) experiments, animals were continuously exposed to light (100 lux) for at least 25 days before the start of behavioral experiments.

Interval timing protocol

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. 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 PI training, the stimulus was a 50-lux light mounted at the center-top of the front wall.

Animals were trained in three consecutive phases: operant lever-press training; FI training; and PI training (Cheng & Meck, 2007; Drew *et al.*, 2007). In all segments of the experiment, sessions occurred once per day, 5 days per week (Monday–Friday).

Operant 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. The lever was retracted after the 20th reinforcement, extended again after a variable delay, and then the cycle was repeated, in order to familiarize mice with the retraction and extension of the lever. After 5 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 re-extended after a variable inter-trial interval (ITI). The session ended when the mouse earned 60 reinforcements or 1 h had elapsed, whatever happened first. After another 5 days of these kind of sessions, mice underwent FI training.

FI training

Lever presses were not reinforced until after a FI had elapsed. Mice received a FI-24s 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 ITI. Trials were separated by a 10–110-s uniformly distributed random ITI. The 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-24s before moving them to the PI training.

PI training

After the FI training, mice received 24 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 three times longer than the FI time, i.e. 96 s, before being terminated. PI trials and FI trials were ordered randomly, with the restriction that no more than five PI trials could occur consecutively. Trials were separated by a 10–110-s uniformly distributed random ITI. The session duration was 90 min. All animals received 24 PI sessions.

Experimental groups

Animals were divided randomly into two groups. The first group of mice (n = 10) was maintained under LD conditions, and was trained and tested for interval timing in the middle of their nocturnal phase at Zeitgeber time (ZT) 15–17. By convention, ZT 12 is defined as the time of lights off. Training and testing time was chosen according to our previous results (Agostino *et al.*, 2011a), which indicate that mice show higher accuracy for interval timing during the night. The second group (n = 10) was maintained under LL conditions, and was trained and tested for interval timing at the same clock hours as their LD controls.

During the FI training, animals from both groups were given a daily i.p. injection of 30/7.5 mg/kg commercially available levodopa/carbidopa (Lebocar 100/25; Pfizer-Pharmacia, Argentina) dissolved in physiological saline or vehicle (saline) 30 min before training (average injection volume 0.132 ± 0.008 mL). Administration type (i.p.) and pretreatment period for levodopa was obtained from previously published data for motor performance tests in mice (Allen *et al.*, 2011). We increased the previously published dose (15 mg/kg, i.p.) in order to induce a larger increment in DA levels. For this purpose, we used the minimal dose that did not have any motor effects (30 mg/kg, i.p.).

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 mouse. The number of responses (in 1-s bins) was averaged daily over trials, to obtain a mean response rate for each mouse. Daily mean response-rate functions for the interval of interest were fit using the Marquardt–Levenberg

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iterative algorithm (Marquardt, 1963) to find the coefficients (parameters) of a Gaussian + linear equation that gave the best fit (least squares minimization) to the data. The following generalized Gaussian + linear model was fit to the individual daily mean response-rate functions: $R(t) = a \times \exp(-0.5 \times [(t - t_0)/b]^2) + c \times (t - t_0) + d$, 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. The parameter $\boldsymbol{t_0}$ (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 (Church *et al.*, 1994), and that differences in these parameters may reflect distinct mechanisms (MacDonald *et al.*, 2012), data were also used to calculate these response thresholds. From the average response rates, start and stop response thresholds (S1 and S2 rate indexes, respectively) were calculated as previously described (Cheng & Meck, 2007; Agostino *et al.*, 2011a, 2013). Briefly, the S1 rate index for the PI-24s 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.

We performed a mixed-design (two-way repeated-measures) ANOVA to analyse S1 and S2 rate indexes across sessions, with lighting conditions as the between-subjects factor.

Single-trial analysis

Analysis of responding in individual peak trials was performed as previously reported (Church *et al.*, 1994; Gallistel *et al.*, 2004; Matell *et al.*, 2006; Balci *et al.*, 2009), with slight modifications. Specifically, the rate of responding on each trial from trial onset to three times the criterion duration was smoothed by calculating a running average with a span of 9 s. The peak time was taken as the point of maximum response. The transition into (start time) and out of (stop time) the high rate was defined by the point at which the smoothed data first exceeded or fell below, respectively, 70% of the maximum response rate. Single-trial analysis was performed only to trials in which mice exhibited 'good timing', that is, response onset prior to the criterion time and response offset following criterion time (Church *et al.*, 1994). Trials with less than three responses were eliminated. This procedure was performed for the last four sessions (sessions 21–24) of PI training. We were able to perform single-trial analysis in three of our four experimental groups. Mice under LL conditions with vehicle administration did not show a clear low–high–low transition.

Molecular procedures

Reverse transcription-polymerase chain reaction (RT-PCR)

Mice under a LD cycle or LL conditions (n = 15/group) were killed by cervical dislocation at either 04:00 h, 12:00 h or 20:00 h, and brains were extracted on ice. These three equally spaced time points were chosen, coinciding with the middle of the night (04:00 h, or ZT 20 for the LD group), the middle of the day (12:00 h, or ZT 4 for the LD group) and the transition day/night (20:00 h, or ZT 12 for the LD group). ZT 12 also coincides with the peak of expression of mouse Period2 (mPER2) in the suprachiasmatic nuclei (SCN), the tissue that was used as a positive control for this biomarker (Field *et al.*, 2000). Total RNA from SCN, dorsal striatum and substantia nigra was isolated in accordance to standard procedures using Trizol reagent (Life Technologies, Gaithersburg, MD, USA). cDNA was synthesized from 3 μg RNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Life Technologies). The following specific oligonucleotide primers were used to amplify mPer2: forward 5′-CGGATGCTCGTGGAATCTTCC-3′ and reverse 5′-GGT TGTGCTCTGCCTCTGTC-3′. Actin expression was used as a housekeeping gene for normalization. PCR reaction was performed under the following conditions: denaturing at 94 °C for 2 min; annealing at 51 °C for 15 s; and primer extension at 72 °C for 30 s in 35 cycles. PCR products were separated by electrophoresis on a 2% agarose gel and stained with GelRed™. An 82-base pair (bp) fragment was amplified for mPer2.

Catecholamine quantification by reverse-phase high-pressure liquid chromatography (HPLC-ED)

Mice under a LD cycle or LL conditions (n = 36/group) were killed by cervical dislocation, and brains were quickly removed and kept at −80 °C. DA and 3,4-dihydroxyphenylacetic acid (DOPAC) levels were determined from the supernatant of homogenized tissue from mouse striatum. Samples were collected every 4 h (n = 6/data point/group). Tissue was homogenized in 1 mL of 0.3 M perchloric acid, centrifuged for 15 min at 3000 $\boldsymbol{g}$ 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 × 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 Quant-it™ Protein Assay kit and the Qubit® fluorometer (Life Technologies).

Western blot

Mice under a LD cycle or LL conditions (n = 15/group) were killed by cervical dislocation at either 04:00, 12:00 or 20:00 h, and brains were quickly removed and kept at −80 °C. Tissue was punched out and homogenized in 50 mM Tris/HCl buffer (pH 7.4), with 0.32 M sucrose, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, a protease inhibitor cocktail (AEBSF, E-64, bestatin, aprotinin and leupeptin) and 2 mM sodium orthovanadate (all drugs from Sigma Chemical, St Louis, MO, USA). Samples were boiled in standard sodium dodecyl sulfate (SDS) sample buffer, and loaded at 30 μg protein per lane onto 9% SDS–polyacrylamide gels. Following separation at 125 V for about 90 min, proteins were transferred onto Hybond nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA), which were then blocked with dried milk in Tween–Tris-buffered saline (TBS; TTBS). After brief washes, membranes were incubated for 24 h at 4 °C with rabbit anti-mouse Per2 (Alpha Diagnostic International, San Antonio, TX, USA; 1 : 1000 in TTBS) or rabbit anti-mouse

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TH antibodies (Millipore, Billerica, MA, USA; 1 : 1000 in TTBS). Immunoreactivity was assessed using a secondary antibody coupled to horseradish peroxidase (Chemicon Int., Temecula, CA, USA; 1 : 5000) and visualized with the ECL kit (GE Healthcare, Piscataway, NJ, USA). Blots were stripped and reincubated for 2 h with anti-α-tubulin antibody (Sigma Chemical; 1 : 1000 in TTBS), and the whole procedure was repeated to visualize the expression of this housekeeping protein.

For molecular experiments, differences between groups were assessed by one-way ANOVA followed by Tukey's test or mixed-design (two-way repeated-measures) ANOVA to analyse biomarker level indices across time, with lighting conditions as the between-subjects factor.

Statistical analysis was performed by using Graphpad Prism (Graphpad Software, San Diego, CA, USA). The alpha level was set at $P < 0.05$ for all statistical analyses.

Results

Effect of levodopa administration on interval timing

LL conditions induce period lengthening followed by circadian arrhythmicity in mice (Meng *et al.*, 2010). Thus, while mice that were entrained to a 12 : 12 h LD cycle exhibited robust wheel-running activity rhythms, mice under LL conditions became arrhythmic (Fig. S1). Previous results from our group indicate that conditions of circadian arrhythmicity due to LL exposure produced a total loss of temporal control in mice in the PI training. In order to elucidate if a transient increase in DA availability could improve time estimation performance under the PI training in mice with circadian arrhythmicity, levodopa/carbidopa (30/7.5 mg/kg, i.p.) or vehicle (saline) were injected daily 30 min before the timing task. Levodopa administration started at the beginning of FI training. During operant lever-press training, animals under an LD cycle or under LL conditions received 10 continuous-reinforcement schedule sessions to learn the association between lever press and the reward delivery. There were no differences in the speed (number of sessions) with which this response was acquired ($F_{4,72} = 1.23$, $P = 0.31$, mixed-design ANOVA for the last five sessions of operant lever-press training) or the number of total lever presses ($t_{18} = 0.30$, $P = 0.77$, two-tailed t-test, $n = 10$/group). During FI training, 24 s was used as criterion time; also during this phase we started daily administration of levodopa or vehicle. When tested during the night, mice under an LD cycle demonstrated an increase of temporal control along sessions, and levodopa administration did not significantly affect timing performance during FI training ($F_{5,40} = 6.35$, $P = 0.0002$ for sessions, $F_{1,40} = 0.03$, $P = 0.86$ for treatment, mixed-design ANOVA comparing the mean S1 rate index along six blocks of three sessions each, $n = 5$/group). In contrast, the group trained under LL conditions with vehicle injection failed to exhibit temporal control of their responses after 18 sessions of 24 s FI training ($F_{5,20} = 0.50$, $P = 0.77$, one-way ANOVA comparing the mean S1 rate index). Under LL, however, levodopa administration led to a significant increase in the mean S1 rate index during FI training ($F_{1,40} = 4.41$, $P = 0.042$ for treatment, mixed-design ANOVA).

The mean proportion of maximum response rate plotted as a function of time for the last session block (sessions 21–24) of PI training is shown in Fig. 1. Mice under an LD cycle focused their response close to the criterion time, reaching a Gaussian-shaped response function (Fig. 1A and B for vehicle and levodopa administration, respectively). There were no significant effects of levodopa on the representative parameters (peak height, peak width and peak

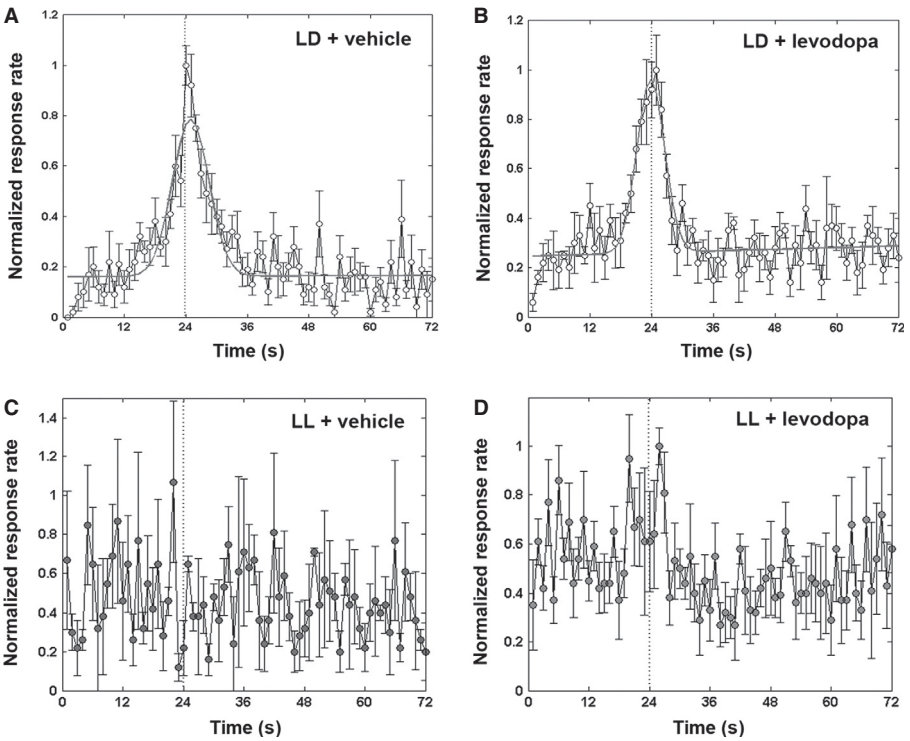

FIG. 1. Effect of levodopa administration on interval timing. Normalized response rate of PI trials as a function of time. The data correspond to mice trained and tested during the night portion of the light/dark (LD) cycle at ZT 15–17, with (A) vehicle or (B) levodopa administration, 30 min before the task. The last four-session block (sessions 21–24) of PI training is shown. The straight line indicates curve fitting to the experimental data. (C and D) The normalized response rate in mice trained and tested under constant light (LL) conditions, with vehicle or levodopa administration, respectively. Data are expressed as mean ± SEM ($n = 5$/group).

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location) obtained by fitting these Gaussian curves ($t_8 = 0.49$, $P = 0.63$ for peak height, $t_8 = 1.88$, $P = 0.10$ for peak width, and $t_8 = 0.72$, $P = 0.49$ for peak location, two-tailed *t*-test for the last session block of PI training, $n = 5$/group). On the contrary, mice under LL conditions showed a total loss of temporal control and were unable to learn the timing task (Fig. 1C). Their response rate along the duration of the trial failed to produce the typical Gaussian-shaped mean response function. However, in mice under LL conditions, levodopa administration revealed an improvement in timing performance, with the response rate increasing around the 24 s post-signal (Fig. 1D). Levodopa treatment did not significantly affect the amount of absolute response rates in any group ($F_{1,16} = 1.51$, $P = 0.24$ for treatment, $F_{1,16} = 0.85$, $P = 0.37$ for group, two-way ANOVA, $n = 5$/group; Fig. S2).

The S2 rate index was used to evaluate learning of the stop response after the criterion time (Cheng & Meck, 2007; see Materials and methods). Figure 2A shows the comparison of the mean S2 rate index along sessions for mice with vehicle or levodopa injection under LD or LL conditions, respectively. Animals under an LD cycle with both vehicle or levodopa administration gradually learned to stop responding after the criterion time with similar performances ($F_{5,40} = 31.36$, $P < 0.0001$ for sessions, $F_{1,40} = 2.85$, $P = 0.10$ for treatment, mixed-design ANOVA). On the other hand, mice under LL conditions treated with vehicle exhibited very low values of the S2 rate index along the sessions, indicating a poor learning of the stop response. However, mice treated with levodopa revealed an S2 rate index with a positive slope that reached higher values compared with mice treated with vehicle ($F_{5,40} = 3.35$, $P = 0.0128$ for sessions, $F_{1,40} = 4.60$, $P = 0.0381$ for treatment, mixed-design ANOVA). Additionally, analysis of the mean S1 rate index indicated no effect of levodopa in either group, LD or LL (LD: $F_{5,40} = 8.10$, $P < 0.0001$ for sessions, $F_{1,40} = 2.07$, $P = 0.16$ for treatment; LL: $F_{5,40} = 0.79$, $P = 0.57$ for sessions, $F_{1,40} = 0.81$, $P = 0.37$ for treatment, mixed-design ANOVA, data not shown). These results suggest that levodopa treatment improved the stop of responding acquisition (S2 response) in mice under conditions of circadian arrhythmicity. Finally, single-trial analysis from the last four sessions of PI training (sessions 21–24) revealed no significant differences in start, stop or peak times among LD + vehicle, LD + levodopa and LL + levodopa groups ($F_{2,12} = 0.19$, $P = 0.83$ for start time, $F_{2,12} = 2.88$, $P = 0.10$ for stop time, and $F_{2,12} = 1.13$, $P = 0.35$ for peak time, one-way ANOVA; Fig. 2B). In this case, the percentage of trials with 'good timing' (see Materials and methods) in mice under LL conditions was lower than in mice under a LD cycle (48.6 ± 8.3 and 69.0 ± 8.2, respectively, data expressed as mean ± SD). Levodopa administration did not induce an increase in the percentage of this type of trials (data not shown).

To investigate the possibility that the deficits in learning the timing task under LL could be related to the abnormality of locomotor activity or anxiety levels, we used both the open-field and elevated plus maze tests to examine animal locomotor activity levels and anxiety levels. In the open-field, mice under LL conditions showed a slightly but not significant reduction in the time spent in the center of the arena, and exhibited similar locomotor activity compared with animals under LD conditions ($t_{16} = 1.50$, $P = 0.15$ for time in the center, and $t_{16} = 0.93$, $P = 0.37$ for locomotor activity, two-tailed *t*-test, $n = 9$/group; Fig. S3A and B). Moreover, elevated plus maze behavior in the functionally arrhythmic group was not different from mice under LD conditions ($t_{24} = 0.42$, $P = 0.68$ for time spent in the open arm, and $t_{24} = 0.58$, $P = 0.57$ for total arm entries, two-tailed *t*-test, $n = 13$/group; Fig. S3C and D). Additionally, we found normal long-term recognition memory performance in the object recognition task ($t_{16} = 1.23$, $P = 0.24$ for retention performance after 6 h, and $t_{16} = 0.92$, $P = 0.37$ for retention performance after 4 days, two-tailed *t*-test, $n = 9$/group; Fig. S4), indicating that cognitive functions are not affected by LL under the conditions of the present study.

Daily oscillation in striatal DA levels

With the purpose to corroborate that the behavioral difference observed in timing behavior under LL conditions was related to differences in DA levels in the striatum, we measured the amount of

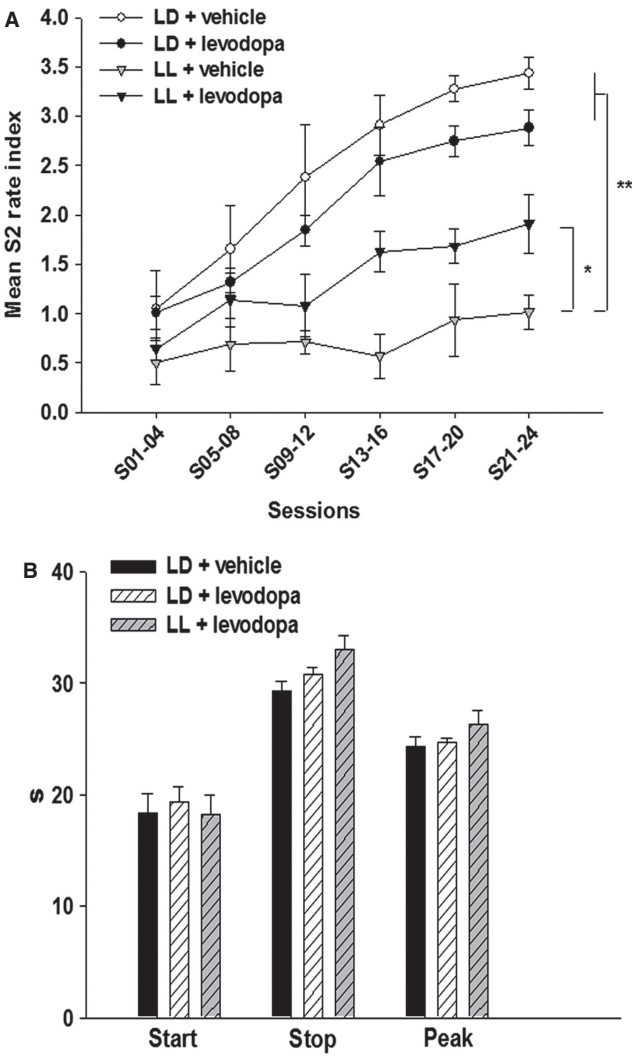

FIG. 2. Quantification of the effect of levodopa administration on interval timing. (A) Mean S2 rate index across sessions for mice under a light/dark (LD) cycle treated with vehicle (white circles) or levodopa (black circles), and mice under constant light (LL) conditions with vehicle (light-gray triangles) or levodopa (dark-gray triangles) administration. The data are expressed as a response-rate ratio for the high rate of responding just after the time of reinforcement (3-s window) divided by the average rate of responding in the second half of the probe trials for PI sessions. $P < 0.0001$ for sessions; $P > 0.05$ for vehicle/levodopa administration for the LD group and $P < 0.05$ for sessions; $P < 0.05$ for vehicle/levodopa administration for the LL group, mixed-design (two-way repeated-measures) ANOVA. (B) Mean start, stop and peak times from single-trial analysis from the last four sessions of PI training (sessions 21–24). No significant differences were found among groups ($P > 0.05$, one-way ANOVA for each parameter). We were not able to perform single-trial analysis with the LL + vehicle group (see Materials and methods). Data are shown as mean ± SEM ($n = 5$/group).

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this neurotransmitter every 4 h under both LD and LL conditions (Fig. 3). Mice under a normal 12 : 12 h LD cycle showed a daily oscillation of DA content in the striatum, with significantly higher levels during the night ($F_{5,30} = 11.84$, $P < 0.0001$, one-way ANOVA followed by Tukey's test, $n = 6$/data point). This result correlates with our previous behavioral data that showed that higher accuracy in time estimation was found in mice trained and tested during the nocturnal portion of the LD cycle (Agostino *et al.*, 2011a), consistent with higher DA levels in the striatum. This daily oscillation of DA levels did not persist under LL conditions ($F_{5,30} = 2.41$, $P > 0.05$, one-way ANOVA), indicating that LL caused circadian disruption in the striatal DA rhythm. Therefore, the lack of accuracy in interval timing under circadian arrhythmicity may correlate with the disappearance of a DA rhythm in the striatum. Taken together, these results suggest that a striatal DA rhythm is necessary for time estimation, and that higher DA levels are associated with more accurate time estimation during the night.

Daily oscillation in DA synthesis and turnover

A critical protein for dopaminergic signaling is TH, the rate-limiting enzyme in DA synthesis (Nagatsu *et al.*, 1964). A daily variation in TH protein levels was found in both the striatum and substantia nigra under LD conditions, and there was a significant effect of LL on this variation. Thus, TH in the dorsal striatum presented a significant daily oscillation, with higher levels during the late night ($F_{2,12} = 17.76$, $P = 0.0003$, one-way ANOVA followed by Tukey's test, $n = 5$/data point; Fig. 4A, black bars). This oscillation was not maintained under LL conditions ($F_{2,12} = 1.21$, $P = 0.33$, one-way ANOVA; Fig. 4A, gray bars). In the substantia nigra, TH levels were higher during the day and early night ($F_{2,12} = 4.84$, $P = 0.0288$, one-way ANOVA followed by Tukey's test; Fig. 4B, black bars), and this daily oscillation did not persist under LL conditions ($F_{2,12} = 0.10$, $P = 0.91$, one-way ANOVA; Fig. 4B, gray bars).

Interestingly, DA turnover in the striatum, expressed as DOPAC/DA levels, also presented a significant rhythm under LD conditions ($F_{5,30} = 2.92$, $P = 0.0291$, one-way ANOVA followed by Tukey's test, $n = 6$/data point; Fig. 4C), which was abolished under LL conditions ($F_{5,30} = 1.14$, $P = 0.36$, one-way ANOVA; Fig. 4D).

Taken together, these results indicate that, under conditions of circadian arrhythmicity, the daily oscillation of both DA synthesis and turnover is eliminated, which may in turn explain the lack of DA rhythmicity in the striatum under LL conditions.

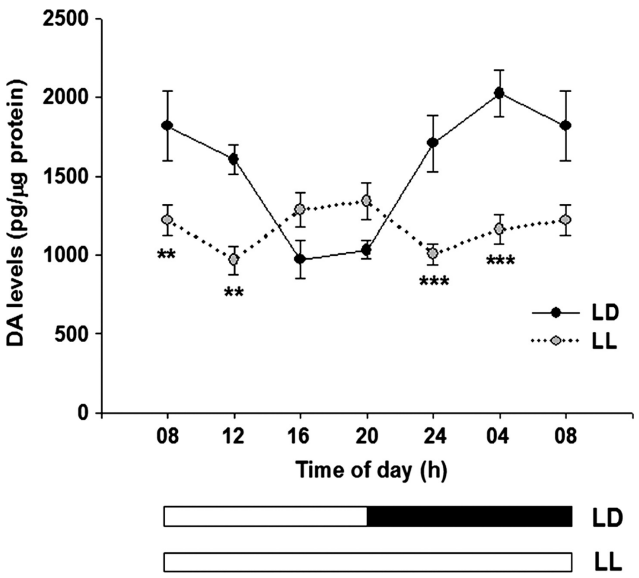

FIG. 3. Daily striatal dopamine (DA) levels in C57BL/6 mice. DA was measured by HPLC-ED. Samples were taken every 4 h from mice under a light/dark (LD) cycle (black circles) or constant light (LL) conditions (gray circles). $P < 0.0001$ for time of day; $P < 0.0001$ for groups, $***P < 0.001$, $**P < 0.01$, two-way ANOVA followed by Bonferroni's test. In addition, one-way ANOVA followed by Tukey's test revealed significant differences across the day for the LD group ($P < 0.001$, 04 vs. 16 and 20 h, $P < 0.01$, 08 vs. 16 and 20 h, $P < 0.05$, 24 vs. 16 and 20 h) but not for the LL group ($P > 0.05$). Data are expressed as mean ± SEM ($n = 6$/data point).

mPer2 expression oscillates in the striatum and substantia nigra

DA signaling has been linked to circadian clock components such as Per2. In this sense, mPER2 has been implied in the circadian regulation of DA metabolism and mood-related behaviors (Hampp *et al.*, 2008). We studied the daily variation of mPer2 expression in the dorsal striatum and substantia nigra, two brain regions relevant for interval timing. Samples were taken every 8 h from mice under LD or LL conditions, and the relative amount of mPer2 RNA was measured by RT-PCR. A rhythmic expression in mPer2 was found in both the dorsal striatum and substantia nigra from mice under LD conditions (Fig. 5). mPer2 expression in the dorsal striatum presented a significant daily oscillation, with higher levels during the day ($F_{2,12} = 6.47$, $P = 0.0124$, one-way ANOVA followed by Tukey's test, $n = 5$/data point; Fig. 5A, black bars). This oscillation was not maintained under LL conditions ($F_{2,12} = 1.72$, $P = 0.22$, one-way ANOVA; Fig. 5A, gray bars). In substantia nigra, mPer2 expression was higher during the night ($F_{2,12} = 35.97$, $P < 0.0001$, one-way ANOVA followed by Tukey's test; Fig. 5B, black bars), and this daily oscillation was not maintained under LL conditions ($F_{2,12} = 0.96$, $P = 0.41$, one-way ANOVA; Fig. 5B, gray bars). mPER2 protein levels also oscillated in the striatum and substantia nigra ($F_{2,12} = 4.51$, $P = 0.034$ for striatum, and $F_{2,12} = 7.32$, $P = 0.0084$ for substantia nigra, one-way ANOVA followed by Tukey's test; Fig. 5C and D, respectively, black bars). Again, LL exposure eliminated mPER2 protein rhythms in both brain areas ($F_{2,12} = 2.28$, $P = 0.14$ for striatum, and $F_{2,12} = 0.99$, $P = 0.41$ for substantia nigra, one-way ANOVA; Fig. 5C and D, respectively, gray bars).

Taken together, our results indicate that under conditions of circadian arrhythmicity, the daily oscillation of Per2 (both at mRNA and protein levels) is eliminated in two brain areas relevant for interval timing, dorsal striatum and substantia nigra. In this sense, PER2 signaling could contribute to DA rhythmicity in the striatum, linking circadian components to pathways related to interval timing.

Discussion

Precise timing is ubiquitous, and of great importance for physiology and behavior. In the seconds-to-minutes range, interval timing is involved in a number of fundamental behaviors, such as foraging, decision-making and learning, via activation of cortico-striatal circuits (Matell & Meck, 2004; Buhusi & Meck, 2005). Organisms are also affected by daily variations of many physical factors of their environment, thus exhibiting circadian rhythms with periods close to 24 h. The circadian system regulates a large array of physiological, metabolic and behavioral functions (Dunlap *et al.*, 2004; Bass & Takahashi, 2010), including performance in several cognitive tasks (Eckel-Mahan & Storm, 2009; Valdez *et al.*, 2010).

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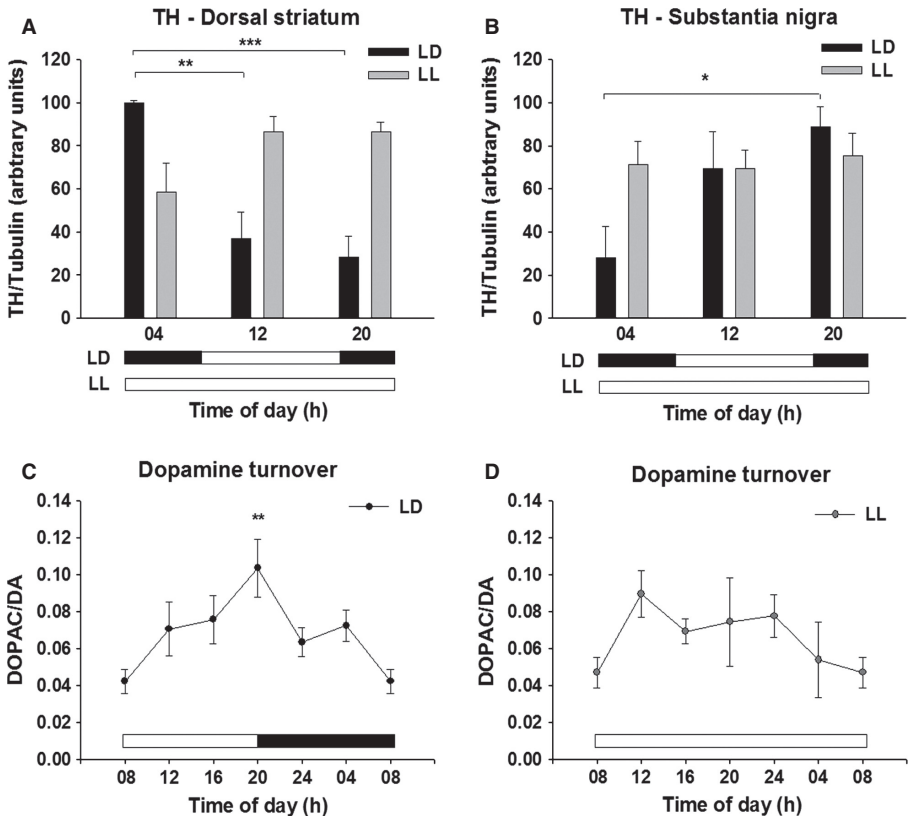

FIG. 4. Daily oscillation in dopamine (DA) synthesis and turnover. Tyrosine hydroxylase (TH) protein levels across the day in (A) the striatum and (B) the substantia nigra from mice under a light/dark (LD) cycle (black bars) or constant light (LL) conditions (gray bars). Data are expressed as mean ± SEM (n = 5/data point). *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA followed by Tukey's test. (C and D) DA turnover in the striatum [expressed as 3,4-dihydroxyphenylacetic acid (DOPAC)/DA levels] under LD or LL conditions, respectively. Data are expressed as mean ± SEM (n = 6/data point). **P < 0.01, one-way ANOVA followed by Tukey's test.

Several studies in different species suggest a close relationship between interval and circadian timing. In particular, the perception of short intervals in humans (Aschoff, 1998; Nakajima *et al.*, 1998; Morofushi *et al.*, 2001), rats (Shurtleff *et al.*, 1990) and mice (Agostino *et al.*, 2011a) changes along the day. It was also reported that sleep deprivation influences diurnal variation of time estimation in humans (Soshi *et al.*, 2010). Moreover, in *Drosophila melanogaster* timing of short intervals is disrupted in circadian mutants for each of the three allelic per mutations, per^1, per^2 and per^3 (Kyriacou & Hall, 1980). Some studies, however, have reported different results for the circadian modulation in interval timing. In particular, Lewis *et al.* (2003) suggested that both systems are independent as they found no effects of SCN lesions on interval timing mechanisms, although the extent of the lesions is not indicated in their work. On the other hand, while the SCN is the master circadian oscillator, there are at least two other circadian oscillators [the food-entrainable oscillator (FEO) and the methamphetamine-entrainable oscillator (MASCO)] that are independent from the primary hypothalamic circadian clock. It was also reported that, when housed under a LD cycle, the Clock mutant mice have no reliable deficits in the accuracy or precision of short time estimation (Cordes & 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 the Clock mutation on interval timing under constant dark conditions. In addition, the neuronal PAS domain protein 2 (NPAS2) acts as a functional substitute for CLOCK in some brain areas, including the basal ganglia (Zhou *et al.*, 1997; Dudley *et al.*, 2003). We have previously reported that both circadian disruption and desynchronization, leading to non-optimal circadian test time, impaired the performance of mice in a 24-s PI timing task (Agostino *et al.*, 2011a). In the present work, we studied the involvement of DA and mPer2 signaling in the interaction between circadian and interval timing.

The PI procedure is a widely used method to test interval timing abilities of many organisms (Catania, 1970; Roberts, 1981; Church *et al.*, 1994; Paule *et al.*, 1999; Matell & Meck, 2004). In this task, averaging data across 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 analysed the mean S1 and S2 rate indexes (Cheng & Meck, 2007). Moreover, we assessed the acquisition of both start and stop responding thresholds using single-trials analysis (Church *et al.*, 1994; Gallistel *et al.*, 2004; Matell *et al.*, 2006). It has been shown that rats and mice learn to stop responding during unreinforced probe trials over the course of PI training rather than acquiring a S2 response threshold during earlier FI training (Balci *et al.*, 2009; MacDonald *et al.*, 2012; Agostino *et al.*, 2013). In accordance with previous works in which levodopa treatment improved temporal production performance in patients with Parkinson's disease (Malapani *et al.*, 1998), we demonstrate that daily injections of levodopa improve timing

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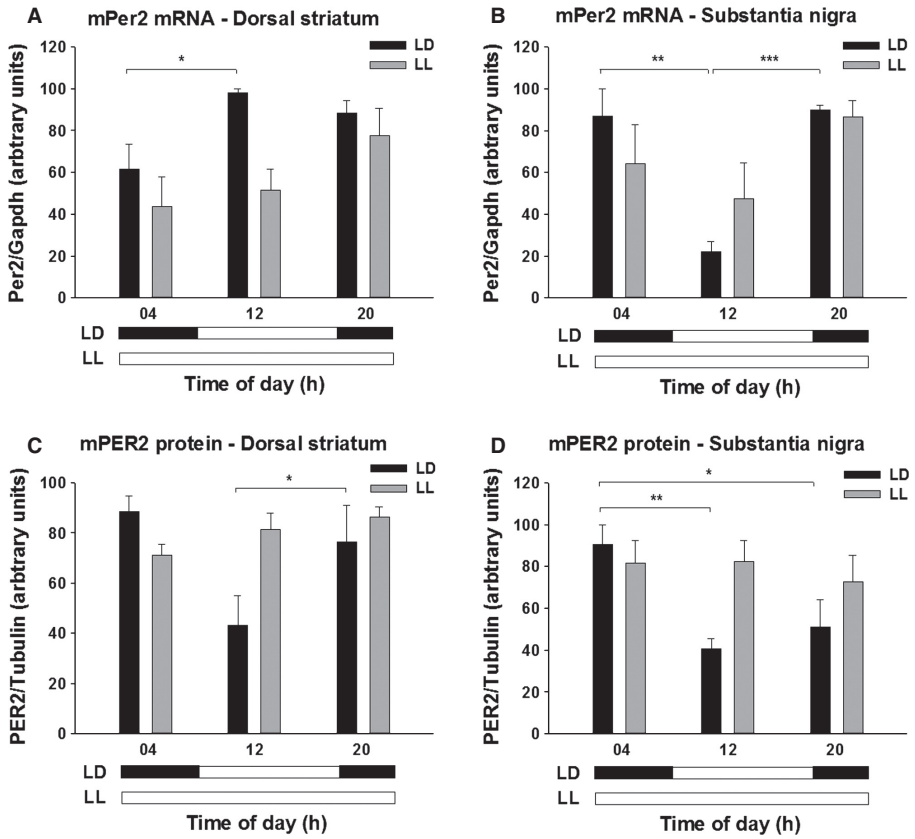

FIG. 5. Daily variations in mouse Period2 (mPer2) mRNA and protein levels. mPer2 mRNA expression in (A) the dorsal striatum and (B) the substantia nigra from mice under a light/dark (LD) cycle (black bars) or constant light (LL, gray bars) conditions. (C and D) PER2 protein levels across the day in the striatum and substantia nigra, respectively, from mice under a LD cycle (black bars) or LL (gray bars) conditions. Data are expressed as mean ± SEM (n = 5/data point). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, one-way ANOVA followed by Tukey's test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

performance in the PI procedure in mice with circadian disruptions, suggesting that a daily increase of DA is necessary for a correct performance in the timing task. In this sense, recent studies have demonstrated the importance of an optimal dopaminergic function for interval timing. This function refers to an inverted U-shape profile, in which an increase in DA availability causes an increase in clock speed, but increasingly high levels induce a decrease in clock speed and/or disruption in timing mechanisms (Meck *et al.*, 2012). Based on our results, we hypothesize that mice under LL conditions have low striatal DA levels, suggesting that the performance of these animals is located on the left portion of the inverted U-shape curve. Levodopa administration increases striatal DA levels in the striatum, leading these animals closer to the optimal dopaminergic levels on the top of the inverted U-shape curve. On the other hand, mice under a LD cycle tested during the night are located close to the top of the inverted U-shape curve. Because of this, a nocturnal increase in DA availability failed to produce an effect on time estimation in these animals. Thus, our results show that levodopa treatment restores the interval timing ability in mice under LL conditions. The parameters measured for each animal by using single-trial analysis (start, stop and peak time) did not differ from mice under a LD cycle. However, we could not perform this type of analysis in the LL + vehicle group (see Materials and methods). As an alternative, we compared these groups using an analysis of the averaged curves. Mice under LL without levodopa treatment showed a lower mean S2 rate index as compared with the other groups (Fig. 2A), but had no difference in the mean S1 rate index. This result suggests that levodopa treatment did not exert a motivational effect (Balci *et al.*, 2010). Instead, the observed improvement in LL with levodopa administration observed in the acquisition of the stop response (mean S2 rate index) might indicate a different effect of levodopa administration in the dorsal and ventral regions of the striatum (MacDonald *et al.*, 2012). Indeed, LL conditions do not affect other cognitive performance variables, as mice under LL conditions had normal long-term recognition memory performance in the object recognition task (Fig. S4). Although this particular cognitive function was not affected by LL under the conditions of the present study, other working memory or attention ability protocols might shed more light on the effects of LL or DA metabolism on cognitive performance.

Numerous studies with dopaminergic modulators led to the DA-clock hypothesis of interval timing, which suggests that DA levels determine the speed of the internal clock. Under this hypothesis, high DA levels increase the speed of the clock whereas low DA levels decrease it, producing a transitory overestimation or underestimation of time, respectively (Meck, 1996). Our results show that striatal DA levels measured by HPLC-ED present a daily rhythm under LD conditions in mice, with lower levels during the day and a peak during the night. This is consistent with previous reports in rats (Castaneda *et al.*, 2004; Hood *et al.*, 2010). Moreover, higher levels of DA during the night coincide with better performance on interval timing in the nocturnal phase of the LD cycle (Agostino *et al.*, 2011a). This daily variation of DA levels in the striatum was affected by inducing circadian disruption under LL conditions,

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indicating that timing impairment under LL (Agostino *et al.*, 2011a) correlates with DA arrhythmicity. Indeed, the effect of levodopa administration on interval timing under LL may imitate the daily increase of DA levels in the striatum. In this way, the improvement in timing performance by levodopa treatment is consistent with a 'restoration' of the daily increase in DA levels. Furthermore, striatal TH levels and DA turnover also presented daily variations that did not persist under LL conditions. Indeed, under LD conditions, TH levels were also rhythmic in substantia nigra, the main dopaminergic input to the striatum. Taken together, these results suggest that striatal DA arrhythmicity is a consequence of the lack of circadian rhythms in both DA synthesis and degradation.

Under LD conditions, rhythmic oscillation of DA in the striatum might be caused by rhythmic input from the substantia nigra or the ventral tegmental area (VTA), and previous studies have demonstrated the expression of circadian clock genes in these structures (Li *et al.*, 2009; Natsubori *et al.*, 2013; Fig. 6). The protein products of these clock genes act as transcription factors through binding to specific elements in promoter regions, such as E-boxes and RORE elements (Lowrey & Takahashi, 2004). These sequences have been found in the promoter region of components involved in dopaminergic metabolism, such as DAT, DA D1A receptor, TH and monoamine oxidase (MAO), suggesting that the expression of these components is under circadian regulation (Yoon & Chikaraishi, 1992; Kawarai *et al.*, 1997; Weber *et al.*, 2004; Hampp *et al.*, 2008). Our results are consistent with these reports, and indicate that both DA synthesis and turnover are under circadian influence.

Moreover, our data also reveal daily oscillations in PER2, a protein intimately related to the molecular circadian clockwork, in the striatum and substantia nigra. Therefore, PER2 could regulate striatal DA rhythmicity by acting as a transcription factor through E-box sequences in key dopaminergic enzymes such as TH and MAO. In mice, PER2 has been reported to regulate monoamine oxidase A activity in the mesolimbic system (Hampp *et al.*, 2008). Moreover, circadian oscillations of PER2 expression in the rat striatum are modulated by DA through D2 receptors (Hood *et al.*, 2010). In humans, Per2 has a role in regulating striatal D2 receptors availability and in vulnerability for cocaine addiction (Shumay *et al.*, 2012). Although the sampling rate was relatively low, our results point out that PER2 protein levels are higher during the night in the striatum and substantia nigra, consistent with previous reports (Hood *et al.*, 2010). Here we hypothesize that higher levels of mPER2 protein during the late night in the substantia nigra (Fig. 5) act positively on MAO (Hampp *et al.*, 2008), and lead to decreased DA input to the striatum at the beginning of the day. It is also possible that the circadian periodicity of DA in the striatum can be mediated by PER2–TH or PER2–D2 receptor regulatory relationships (Shumay *et al.*, 2012). Figure 6 summarizes the principal molecular mechanisms supporting the interaction between the circadian clock and interval timing. The main dopaminergic input to the striatum comes from the substantia nigra and VTA, and circadian clock genes are expressed in these structures. Circadian proteins bind to E-boxes and ROR elements from promoter regions, thus regulating transcription of target genes. The circadian control of dopaminergic enzymes could be involved either in rhythmic DA synthesis by TH, rhythmic DA release (under control of D2 autoreceptors) or rhythmic degradation mediated by DAT and MAO. Some of them, such as MAO, also exhibit diurnal rhythms in enzymatic activity (Hampp *et al.*, 2008). Moreover, circadian rhythms in DA levels were reported in both the striatum and nucleus accumbens (Castaneda *et al.*, 2004; Hood *et al.*, 2010; see also Fig. 3). Furthermore, diurnal rhythms in dopaminergic transmission were also found in these brain areas. In

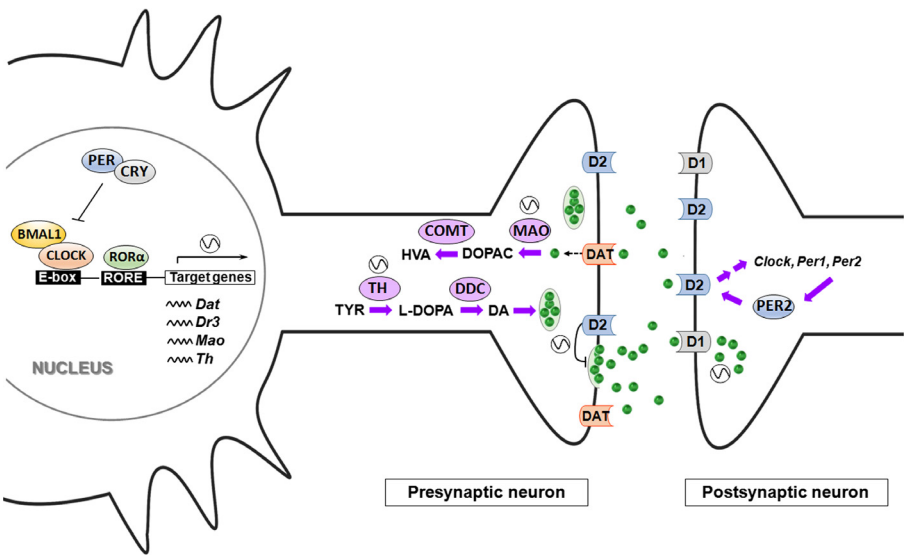

FIG. 6. Proposed model depicting the molecular mechanisms for circadian modulation of interval timing. The circadian system controls dopaminergic transmission at both presynaptic and postsynaptic levels. In presynaptic neurons, circadian clock proteins generate daily rhythms in the expression of components related to dopaminergic neurotransmission, mainly by acting as transcription factors through binding to E-boxes and ROR elements from target promoter regions. Circadian control of dopaminergic enzymes could be involved either in rhythmic DA synthesis by TH, rhythmic DA release (under control of D2 autoreceptors) or rhythmic degradation mediated by DAT and MAO. On the other hand, in postsynaptic neurons (such as striatal medium spiny neurons) there could be multiple levels of control by circadian components. There are daily rhythms in DAT expression, DA content and D2 receptor availability. In turn, dopaminergic function regulates the expression of clock genes through the activation of D2 receptors. Moreover, a polymorphism in the PER2 protein correlates with striatal D2 receptor availability, suggesting that the expression of this receptor in the striatum is regulated by PER2. COMT, catechol-o-methyl transferase; D1, dopamine receptor type 1; D2, dopamine receptor type 2; DA, dopamine; DAT, dopamine transporter; DDC, DOPA decarboxylase; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; MAO, monoamine oxidase; ROR, retinoid-related orphan receptor; RORE, ROR response element; SN, substantia nigra; TH, tyrosine hydroxylase; TYR, tyrosine.

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particular, the expression of DAT and TH showed a daily rhythm; however, this rhythmic expression was abolished in SCN-lesioned rats (Sleipness *et al.*, 2007). There is also recent evidence indicating a 24-h rhythm in the expression of the DA D3 receptor, which is enhanced by circadian elements RORα and inhibited by Rev-Erbα (Ikeda *et al.*, 2013). Additionally, there are reports that implicate the activation of DA receptors through circadian clock proteins in the striatum, particularly the D2 receptor subtype (Shumay *et al.*, 2012), which is closely related with interval timing (Drew *et al.*, 2007). Quinpirole, a D2 receptor agonist, inhibited CLOCK and PER1 expression in primary striatal neurons in culture (Imbesi *et al.*, 2009). Moreover, Hood and colleagues found that blockade of D2 receptors blunted the rhythm of striatal PER2, and daily activation of D2 receptor restored and entrained the PER2 rhythm in DA-depleted striatum in rats (Hood *et al.*, 2010). Reciprocally, a recent study in humans suggests that a polymorphism in the PER2 protein correlates with striatal D2 receptor availability (Shumay *et al.*, 2012). Taken together, these evidences suggest a circadian regulation of dopaminergic transmission in striatal circuits. This interaction at the dopaminergic level could be in part responsible for the interaction between the circadian system and short-time estimation.

Our results suggest that interval and circadian timing might share some common features related to their molecular mechanisms, and might also influence one another. In addition, both timing mechanisms might be affected by neural circuits regulating the activation of reward pathways in the brain, such as those driven by food stimulation in partially deprived animals. It has been shown that restricted daily food access acts as an entraining stimulus for the FEO. Food-entrained circadian rhythms are characterized by increased locomotor activity in anticipation of food availability (food anticipatory activity, FAA). Animals anticipate food at intervals ranging from seconds to days (Silver *et al.*, 2011), and FAA has been shown to occur in studies of interval timing (Balsam *et al.*, 2009). Although the possible correspondence of the brain structures and networks responsible for timed anticipation of short intervals and those that underlie circadian FAA is not known, because the mice in the present study are under the influence of a single daily feeding schedule, this link is worth exploring in the future. In addition, the dopaminergic reward mechanisms that might represent this interval timing–circadian link could also be related to the SCN-independent MASCO (Iijima *et al.*, 2002; Williamson *et al.*, 2008). Daily administration of methamphetamine dramatically lengthened the circadian period of locomotor activity rhythms and induced restoration of these rhythms in mice with SCN ablation (Tataroglu *et al.*, 2006). A recent study showed that methamphetamine-induced phase shift in liver, lungs, salivary and pituitary glands was attenuated by pretreatment with a D1 antagonist (Mohawk *et al.*, 2013). Moreover, methamphetamine administration causes a phase advance in Per2 rhythms in dopaminergic areas such as the striatum and substantia nigra in SCN-intact rats (Natsubori *et al.*, 2014). Taken together, these results suggest that the MASCO is driven by a combination between the circadian and the reward systems.

In summary, our findings add further support to the notion that circadian and interval timing share some common processes, interacting to some extent at the level of the dopaminergic system.

Supporting Information

Additional supporting information can be found in the online version of this article:

Data S1. Materials and methods.

Fig. S1. Assessment of locomotor activity under a light/dark cycle or constant light conditions.

Fig. S2. Absolute response rate of PI trials.

Fig. S3. Anxiety-related behaviors.

Fig. S4. Novel object recognition task.

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Abbreviations

DA, dopamine; DAT, dopamine transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; FAA, food anticipatory activity; FEO, food-entrainable oscillator; FI, fixed-interval; HPLC-ED, reverse-phase high-pressure liquid chromatography; ITI, inter-trial interval; LD, light/dark; LL, constant light; MAO, monoamine oxidase; MASCO, methamphetamine-entrainable oscillator; mPER2, mouse Period2; PI, peak-interval; RT-PCR, reverse transcription-polymerase chain reaction; SCN, suprachiasmatic nuclei; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TH, tyrosine hydroxylase; TTBS, Tween–Tris-buffered saline; VTA, ventral tegmental area; ZT, Zeitgeber time.

Conflict of interest

The authors declare no conflict of interest.

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