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Selective serotonin reuptake inhibitors and raft inhibitors shorten the period of *Period1*-driven circadian bioluminescence rhythms in rat-1 fibroblasts

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Abstract

Alterations in circadian rhythm generation may be related to the development of mood disorders. Although it has been reported that the most popular antidepressant, selective serotonin reuptake inhibitors (SSRIs) affect circadian phase, no data are available that describe the effects of SSRIs on other circadian parameters (period, amplitude and damping rate) in dissociated cells. In the present study we used real-time monitoring of bioluminescence in rat-1 fibroblasts expressing the *Period1-luciferase* transgene, and that in *Period1-luciferase* transgenic mouse suprachiasmatic nucleus (SCN) explants, in order to characterize the effects of SSRI on circadian oscillator function in vitro. We found that mRNA of the serotonin transporter (SERT), a target of SSRIs, was expressed in rat-1 fibroblasts. Sertraline, fluoxetine, fluvoxamine, citalopram and paroxetine all significantly shortened the period of *Period1*-bioluminescence rhythms in rat-1 fibroblasts. The amplitude was reduced by sertraline, and the damping rate was decreased by sertraline, fluoxetine, flvoxamine and paroxetine. The effect of sertraline was dose-dependent, and it also shortened the circadian period in the SCN. SERT is associated with lipid microdomains, which are required for efficient SERT activity. Indeed, cholesterol chelating reagent methyl-β-cyclodextrin significantly reduced the period and the amplitude in rat-1 fibroblasts. Furthermore, lipid binding reagent xylazine significantly reduced the period. In summary our data present evidence that SSRIs affect circadian rhythmicity. The action of SSRIs is likely mediated by suppression of SERT activity. A better understanding of the relationship between mental illness and biological timing may yield new insight into disease etiology and avenues for treatment.

Keywords

SSRI; SERT; Lipid raft; Methyl-β-cyclodextrin; Xylazine; Real-time circadian bioluminescence; monitoring; Fibroblasts; Mouse suprachiasmatic nucleus

Introduction

Circadian rhythms are cyclic variations in biological activity which are generated by internal (circadian) clocks in most living organisms on earth (Dunlap et al., 2003). The molecular circadian clock in mammals has been elucidated over the past decade (Ko and Takahashi, 2006). In mammals, the central circadian clock is localized to the suprachiasmatic nucleus (SCN) of the hypothalamus, which synchronizes circadian functions within the body and is

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necessary for behavioral and hormonal rhythms. Peripheral circadian oscillators have also been identified in many types of cells and tissues (Balsalobre et al.,1998; reviewed in Fukuhara and Tosini, 2003; Brown et al., 2005; Yamazaki et al., 2000; Yoo et al., 2004; Welsh et al., 2004). The expression patterns of circadian clock genes and proteins are similar between SCN neurons and peripheral tissues (Ko and Takahashi, 2006), suggesting that the circadian machinery is similar between the central (SCN) and peripheral cells.

Light is the most effective naturally occurring time cue to entrain the central circadian clocks to the exact 24-hour cycles. In addition, many chemicals affect circadian rhythm generation by shifting clocks and/or affecting period length. For example, lithium lengthens period of SCN neuronal rhythms and behavioral rhythms in many organisms (Klemfuss, 1992). In a previous study, we have shown that SP600125, an inhibitor of one of the mitogen-activated protein kinases (MAPKs) c-Jun N-terminal kinase (JNK), similarly extends period in fibroblasts and the mouse SCN (Chansard et al., 2007). In addition, valproic acid shortens the period in rat-1 fibroblasts (Chansard et al., 2007). These results further suggest that peripheral cell models have a potential to allow us to study the effects of chemicals on circadian rhythm generation. Indeed, the fibroblast model has been used to record circadian rhythms in rodents and even humans (Balsalobre et al., 1998; Brown et al., 2005; Chansard et al., 2007). This model also enables us to reduce the number of animals to be used.

It has been suggested that alterations in circadian rhythm generation may underlie the development of mood disorders; for example, affected phases have been reported to correlate with depression and bipolar disorders (Ehlers et al.,1988; Grandin et al., 2006; Lader, 2007; Malkoff-Schwartz et al., 2000; McClung, 2007). Incidentally, some therapeutic treatments, such as some antidepressants, total sleep deprivation, and bright light therapy have been shown to affect circadian rhythm generation. One of the most popular antidepressant, selective serotonin reuptake inhibitors (SSRIs) have been reported to affect circadian phase in vivo and in vitro (Gannon and Millan, 2007; Prosser et al., 2006; Sprouse et al., 2006). However, no data are available that describe the effects of these agents on circadian parameters (period, amplitude and damping rate) in dissociated cellular oscillator organizations in periphery.

In the present study, we used real-time monitoring of bioluminescence in rat-1 fibroblasts, stably transfected with *Period1-luciferase* (*Period1* promoter; one of circadian clock genes) reporter gene (Chansard et al., 2007) as well as *Period1-luciferase* transgenic mouse SCN explants to test any effects that SSRIs might have on the circadian rhythm generation. Since SSRI inhibits serotonin reuptake by inhibiting the activity of serotonin transporter (SERT), we first confirmed that SERT mRNA is expressed in rat-1 fibroblasts. Results of real-time-bioluminescence recording suggest that SSRI significantly shortened the period in rat-1 fibroblasts and the mouse SCN. SERT is associated with lipid microdomains, which are required for efficient serotonin transport activity (Magnani et al., 2004). Therefore, to further support our findings, SERT activity was blocked using a cholesterol chelating reagent methyl-β-cyclodextrin (β-CD) and lipid binding reagent xylazine. Both chemicals significantly shortened the period in rat-1 fibroblasts.

Materials and methods

Animals

Transgenic mice carrying a *Period1-luciferase* transgene as a reporter of activity were generously provided by Dr. Hajime Tei (Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan). *Period1-luciferase* mice were maintained at Morehouse School of Medicine. All animals were housed under a 12-hour light:12-hour dark cycle (light on, 0700–1900) in a temperature-controlled environment and had access to food and water ad libitum. Zeitgeber time (ZT0) was defined as light onset and ZT12 as light offset. Wild type siblings were used

for RT-PCR experiment. Both heterozygous and homozygous mice were used in our bioluminescence recording experiments. All experiments were run under the oversight of IACUC of the Atlanta University Center and NIH guidelines.

Drugs

Sertraline hydrochloride, fluvoxamine maleate, paroxetine hydrochloride, methyl-β-cyclodextrin, and xylazine were purchased from Sigma-Aldrich, St Louis, MO, USA. Fluoxetine hydrochloride and citalopram hydrobromide were purchased from TOCRIS Bioscience, Ellisville, MO, USA.

Amplification of serotonin transporter (SERT) mRNA expression

An adult male wild type mouse was sacrificed by decapitation under CO_2 anesthesia. A whole brain was removed from the animal and the brain was kept at −80 °C until RNA extraction. Detailed procedures for RNA extraction and RT-PCR methods were described in our previous study (Fukuhara et al., 2002). PCR was carried out using following primers: *Sert*, sense: 5′-ATCATAGCCTGGGCGCTCTAC-3′, antisense: 5′-CATGTAGCCAAGCACCGTGAA-3′ ([Ushijima et al., 2005], GenBank accession no. AF013604) and *Gapdh*, sense: 5′-AGACAGCCGCATCTTCTTGT-3′, antisense: 5′-TGATGGCAACAATGTCCACT-3′ (GenBank accession no. X02231). Thirty seven cycles of amplification were carried out following 30 s of denaturation at 94 °C. Once the temperature reached 94 °C, it was decreased to 57 °C for 10 s, and raised to 72 °C for 40 s The PCR products were separated by electrophoresis through a 3% agarose gel.

Rat-1 fibroblasts culture

Detailed procedures are described in the previous study (Chansard et al., 2007). In brief, 3 days before starting experiments, 10^6 rat-1 fibroblasts, stably transfected with *Period1-luciferase* reporter gene ([Izumo et al., 2003], generous gift from Carl Johnson, Vanderbilt University, Nashville, TN, USA), were seeded in a 35 mm Petri dish. Cells were cultured at 36 °C under 5% CO_2–95% air atmosphere in 2mL of Dulbecco's Eagle Modified Medium (Gibco, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum,100 units/mL penicillin and 100 μg/mL streptomycin. For all experiments, circadian rhythms were synchronized among the cells by replacing the culture medium with 2 mL of assay medium (Hirota et al., 2002), constituted of serum-free DMEM, 10 mM HEPES, 350 mg/mL sodium bicarbonate, 2% B27 supplement, 25 units/mL penicillin, and 25 μg/mL streptomycin. For bioluminescence analysis, 0.1 mM luciferin was added to the assay medium. Sertraline, fluvoxamine, paroxetine, fluoxetine, citalopram, and xylazine were added when medium was replaced with the assay medium. 30 μM of each chemical was used to test the effects on circadian parameters, because the μM range of SSRIs has been successfully used in the previous circadian studies (Prosser et al., 2006; Sprouse et al., 2006). Cells were pre-incubated with methyl-β-cyclodextrin (β-CD) for 24 h before medium was replaced with the assay medium. β-CD or xylazine was continuously supplied in the assay medium while cells were placed in the Lumicycle apparatus.

SCN tissue culture

Detailed procedures are described in the previous study (Chansard et al., 2007). In brief, the *Period1-luciferase* mice were sacrificed by decapitation under CO_2 anesthesia at ZT3. A brain was removed from animal and dissected at 125-micrometer thickness using a vibratome (World Precision Instruments, Sarasota, FL, USA). The brain section containing the SCN was further dissected as 1 mm bottom × 1 mm height triangle. Each piece of SCN was cultured individually on Millicell culture membranes (Millipore, Billerica, MA, USA) placed in 35 mm culture dishes. The SCN was cultured for 3 days before monitoring bioluminescence levels at 36 °C

under 5% CO_2–95% air atmosphere. At ZT7 the medium was changed to fresh assay medium, and dishes were placed in the Lumicycle.

Bioluminescence monitoring and data analysis

As shown in our previous study (Chansard et al., 2007), each chemical or their solvent as their vehicle was added in the recording medium, and dishes were sealed using cover slips and grease. The samples were placed in a real-time-bioluminescence monitoring system (Lumicycle, Actimetrics, Wilmette, IL, USA) placed in a 36 °C air incubator. Photons emitted from cells in each dish were monitored by photomultipliers for 60 s at 10-minute intervals. The data were acquired using a PCI-6601 card and a NI-DAQ 7.1 acquisition software (Lumicycle Software, Actimetrics).

Data analyses were performed using the Lumicycle Data Analysis Software (version 2.10, Actimetrics) and OriginPro (version 7, Origin Lab, Northampton, MA, USA). First, a chi-square periodogram analysis were performed on each baseline-subtracted data to establish whether or not there was a significant circadian rhythm ($P<0.05$; Sokolove and Bushell, 1978). Using Lumicycle software the raw data were subjected to baseline correction, performed by μ a polynomial curve to the data. The baseline fit curve obtained was subtracted from the raw data and the resulting baseline-subtracted data was used to determine the period and amplitude. The damping rate was defined as the number of days necessary for the amplitude of the rhythm to decrease to $1/e$ (≈ 36.79%) of its initial value. Using OriginPro, the raw data were 2 h smoothed. For the period calculation, the second peak and fourth peak were chosen and split by 3 days.

The baseline level of bioluminescence, which corresponds to the background level, was evaluated at 28.79 ± 7.66 counts/s.

Statistics

Data are expressed as means ± SEM, and to obtain statistical significance each value was subjected to parametric and non-parametric statistics.

Results

Sert mRNA expression

RT-PCR was first performed to detect the mRNA expression of *Sert* in rat-1 fibroblasts. As a positive control, mouse whole brain was used to amplify *Sert* mRNA expression (Ushijima et al., 2005). In both rat-1 fibroblasts and whole brain, expected sizes of *Sert* and *Gapdh* DNA fragments were amplified as single bands (Fig. 1).

The effects of SSRIs in rat-1 fibroblasts

Since *Sert* mRNA was expressed in rat-1 fibroblasts, we then examined the effects of SSRIs on circadian parameters; period, amplitude, and damping rate, in rat-1 fibroblasts. As shown in Table 1, all SSRI treatments shortened the period compared with control and their vehicle-treated samples. The amplitudes were affected by sertraline, but not other SSRIs. Damping rate was affected by sertraline, fluoxetine, fluvoxamine and paroxetine treatment (Table 1). Since all SSRIs shortened the period, we further examined the effects of the most popular SSRI, sertraline. Fig. 2A showed the representative record of real-time-bioluminescence rhythms, affected by sertraline. Sertraline dose-dependently shortened the period (Fig. 2B). Although 1 μM sertraline did not significantly shorten the period, 20 μM and 40 μM sertraline shortened the period to 23.59±0.13 h ($P<0.005$ vs control, $P<0.0005$ vs DMSO) and 22.28±0.05 h ($P<0.0005$ vs control, $P<0.0005$ vs DMSO), respectively. Additionally, sertraline affected the amplitude. 20 μM and 40 μM sertraline reduced the amplitude to 109.14±6.76 h ($P<0.005$ vs

control) and 68.77±8.02 counts/s (P<0.0005 vs control, P<0.0005 vs DMSO), respectively. Damping rate also was affected; 20 μM and 40 μM sertraline reduced the damping rate to 1.33 ±0.09 h (P<0.0005 vs control, P<0.05 vs DMSO) and 1.27±0.13 counts/s (P<0.005 vs control, P<0.05 vs DMSO), respectively.

The effects of sertraline in the mouse SCN

To test whether sertraline similarly affected the circadian parameters in the central clock, the effects of sertraline in the mouse SCN were tested (Fig. 3). Control SCN explants showed period (24.39±0.06 h), amplitude (43.39±10.34 counts/s), and damping rate (2.78±0.43 days) that are similar to those previously reported (Chansard et al., 2007), whereas 5 μM sertraline significantly shortened the period (23.38± 0.23 h, P<0.005, Fig. 3B) without affecting amplitude (33.72± 9.86 counts/s, P>0.05) and damping rate (3.09±0.24 days, P>0.05).

The effects of lipid raft inhibition in rat-1 fibroblasts

Cholesterol is a major component of lipid rafts. As the association of SERT with lipid rafts may represent a mechanism for regulating the SERT activity (Scanlon et al., 2001; Magnani et al., 2004), we further used a cholesterol chelating reagent β-CD to analyze the effects of cholesterol depletion, consequently the inhibition of SERT activity, on circadian rhythms. Our preliminary results showed that 0.5, 0.8, and 1 mM β-CD shortened the period, but cells were not able to survive in the presence of 2 mM β-CD. Therefore, we used 1 mM β-CD for further experiments. As shown in Fig. 4 β-CD significantly shortened the period to 22.49±0.21 h (P<0.0005), and reduced the amplitude to 60.46±6.77 counts/s (P<0.0005), without affecting damping rates.

We also examined the effects of a non-specific lipid binding reagent xylazine, which can inhibit the cell proliferation at the concentration of 100 μg/mL in cultured cells, to further support the β-CD results (Huo et al., 2003). Xylazine (100 μg/mL) treatment significantly shortened the period to 23.47±0.16 h (P<0.005, Fig. 4),whereas 50 μg/mL xylazine did not affect the period (data not shown). Amplitude and damping rate were not affected by 100 μg/mL xylazine treatment (in both cases, P>0.5).

Discussion

In the present study, we found that SSRIs significantly shorten the period, and accelerate damping rate in fibroblasts (Table 1, Figs. 2 and 3). A common target of all the SSRIs is the SERT (Harten,1993). In mouse, we found that *Sert* mRNA is expressed ubiquitously, such as in fibroblasts and whole brain (Fig. 1) as well as the SCN, heart, lung, liver and kidney (data not shown), thus it suggests that SSRI could affect SERT functions in many tissues, organs and cells, but not only in the CNS. Furthermore, since serotonin synthesis has been suggested in mammalian skin, including fibroblasts (Slominski et al., 2003), fibroblasts could represent an appropriate model to test the effects of SSRI. The fact that sertraline similarly shortens the period in the SCN and fibroblasts (Figs. 2A, B and 3). It suggests that fibroblasts can be used to evaluate the effects of SSRIs on circadian oscillator functions.

Although the degree of shortened period length was not significant among the SSRIs, there is a tendency that paroxetine has stronger impact than other SSRIs tested, and fluvoxamine showed the least effects (Paroxetine>fluvoxamine). It is interesting to mention that SSRIs binding affinity to the SERT and the degree of shortened period likely well correlate (Hirano et al., 2005), thus it suggests that the effects of SSRIs on the period are unlikely of a non-specific nature.

We found that damping of *Period1* promoter-driven bioluminescence rhythms in fibroblasts are accelerated by SSRI treatments, except for citalopram (Table 1). Further studies will provide evidence whether such damping is caused by desynchronization of circadian rhythm generation among cells, or damped rhythms within a cell. Since citalopram does not affect the damping rate, it would be of interest to further find if citalopram has any clinical relevance superior to other SSRIs.

Since SSRIs inhibit SERT activity, it is plausible to speculate that SSRIs shorten the period by inhibiting SERT activity. To further support this speculation, the effects of SERT activity suppression by lipid raft and cholesterol binding reagents were further examined (Fig. 4). SERT is an *N*-glycosylated integral membrane protein with 12 transmembrane regions, and has been shown to associate with lipid raft. The association with lipid raft on the cell membrane is required for an efficient 5-HT transporter activity (Magnani et al., 2004). The loss of interaction with lipid microdomains is detrimental to SERT function, possibly due to conformational changes that mainly affect the translocation of the substrate across the plasma membrane. The conformational changes of SERT by cholesterol depletion using β-CD result in the reduction of SERT activity (Scanlon et al., 2001). A nonspecific lipid binding reagent xylazine is another type of chemical that can interrupt proper lipid raft functions (Huo et al., 2003). We found that both chemicals shorten the period (Fig. 4A and B). However, it is worth to note that only β-CD affected the amplitude (Fig. 4C) that is probably due to different mechanisms by which two chemicals affect membrane properties. The observation, taken together with the SSRIs data, suggests that the SSRI likely shortens the period by inhibiting the SERT functions. However, a possibility that other membrane associated molecule, affected by β-CD and xylazine, contributes to the regulation of circadian rhythm generation, cannot be excluded.

The mechanisms how SSRIs shorten the period downstream from the SERT are not yet fully elucidated. One of the potential molecules that mediate the effects of SSRI is glycogen synthase kinase-3 (GSK3). Indeed, fluoxetine has been reported to affect the level of phosphorylated form of GSK3β in the mouse brain (Li et al., 2007). GSK3 has been recognized as an important enzyme that modulates many aspects of neuronal functions, including circadian rhythm generation and mood (Phiel and Klein, 2001; Gould et al., 2006; Yin et al., 2006; Kaladchibachi et al., 2007). Another potential intracellular target by which SSRI affects the period is MAPKs. Chou et al. (2007) have shown in their paper that paroxetine phosphorylates three MAPKs: extracellular- regulated kinase (ERK), JNK, and p38. Previous studies, including our paper have reported that inhibition of JNK or p38 affects the period in mammals, chick, and *Xenopus* (Hayashi et al., 2003; Hasegawa and Cahill, 2004; Chansard et al., 2007). Future studies will provide crucial answers to find if these kinases are recruited to regulate the period upon SSRI treatment.

It has been suggested that affected circadian rhythm generation (sleep–wake cycles) and the development of certain mental illnesses, such as depression, bipolar disorders, and seasonal affective disorders are somehow correlated (Ehlers et al., 1988; Malkoff-Schwartz et al., 2000; Grandin et al., 2006; Lader, 2007; McClung, 2007). A recent study has shown that when total sleep deprivation and light therapy advance phase of activity–rest rhythms in bipolar depression patients, the degree of depression is significantly reduced/improved (Benedetti et al., 2007). Phase advancing effects of these two therapies support the significant relations between circadian rhythm generations and regulation of mood. In addition to the circadian hypothesis, functional deficiencies in the regulation of 5-HT levels have been implicated in the pathophysiology of depressive syndromes, and restoring the normal functions of 5-HT associated signaling pathways has been one of the targets of antidepressants (Heninger et al., 1996). Indeed, the second-generation antidepressants SSRIs have been a major choice in clinical treatment. Our data, presenting evidence that SSRIs affect circadian rhythmicity by suppressing SERT activity, may help better understand the relationship between mental illness

and biological timing to yield new insight into disease etiology and avenues for effective therapeutic treatments.

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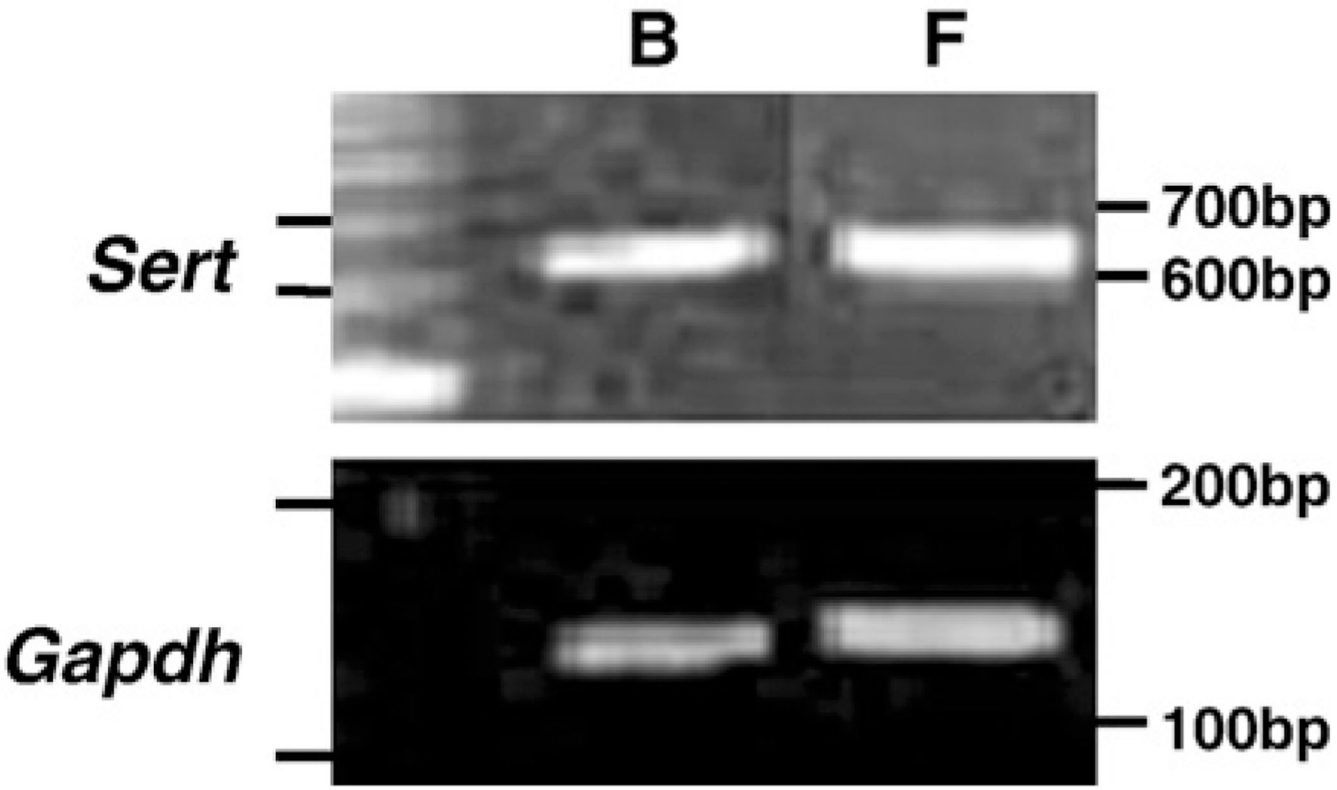

Fig. 1.

Amplification of serotonin transporter (SERT) in rat-1 fibroblasts and mouse whole brain. Total RNA isolated from rat-1 fibroblasts (F) and mouse whole brain (B) were subjected to RT-PCR to amplify *Sert* and *Gapdh*. Ethidium bromide-stained agarose gel indicates amplicons for *Sert* (614 bp) and *Gapdh* (142 bp) in both samples.

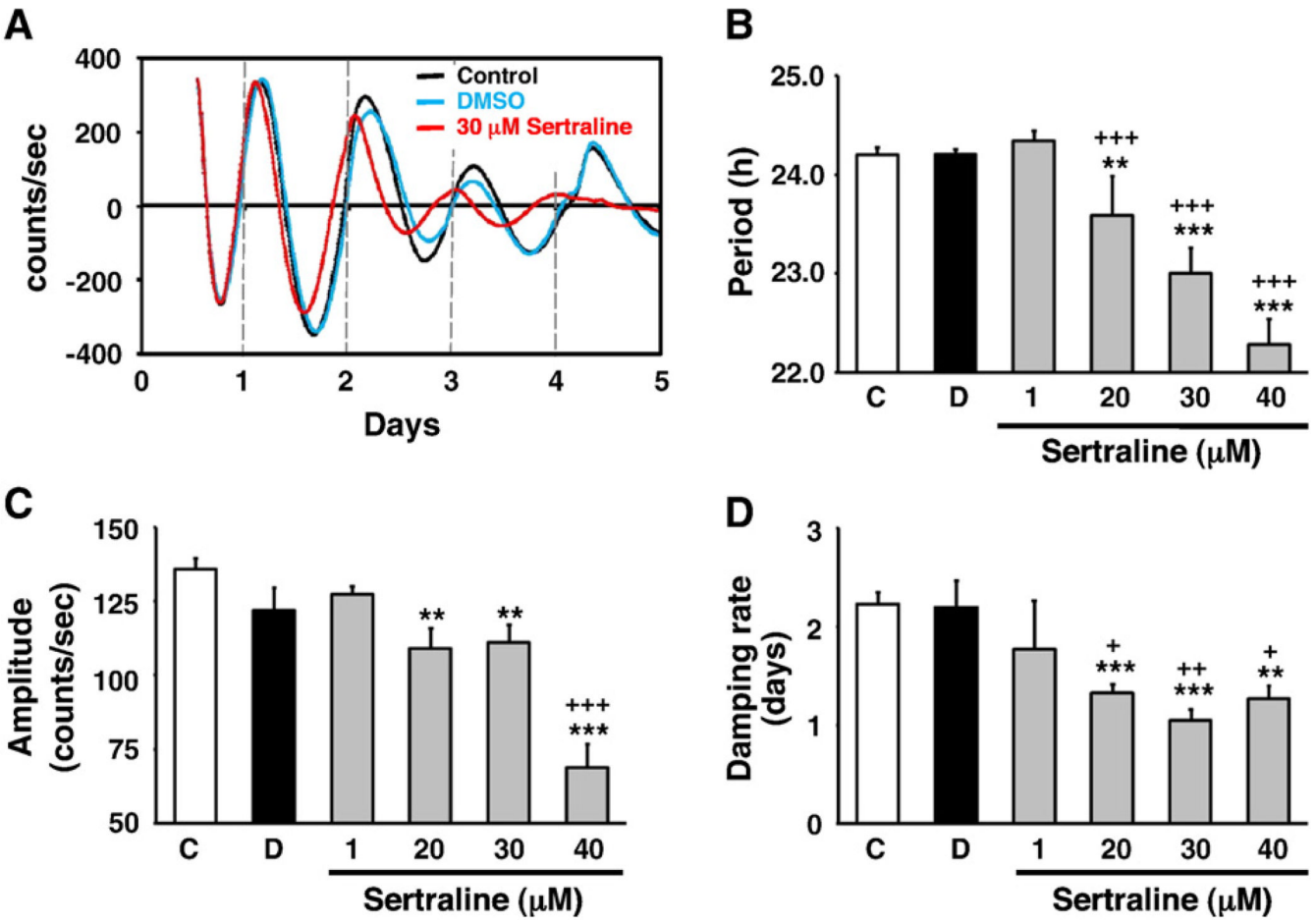

Fig. 2.

The effects of sertraline in rat-1 fibroblasts. (A) Synchronized *Period1*-driven bioluminescence rhythms were monitored in the presence of sertraline in rat-1 fibroblasts. Representative baseline normalized records for control (black), vehicle (DMSO, blue), and sertraline (red) are shown. 30 μM sertraline significantly affected the period, amplitude and damping rate. (B–D) The dose-dependent effects of sertraline (1–40 μM) on the circadian period (B), amplitude (C) and damping rate (D). C, Control; D, DMSO. Values are means±SEM (N = 4, from two independent experiments).**, P<0.005; ***, P<0.0005 (Control vs); +, P<0.05; ++, P<0.005; +++, P<0.0005 (DMSO vs). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

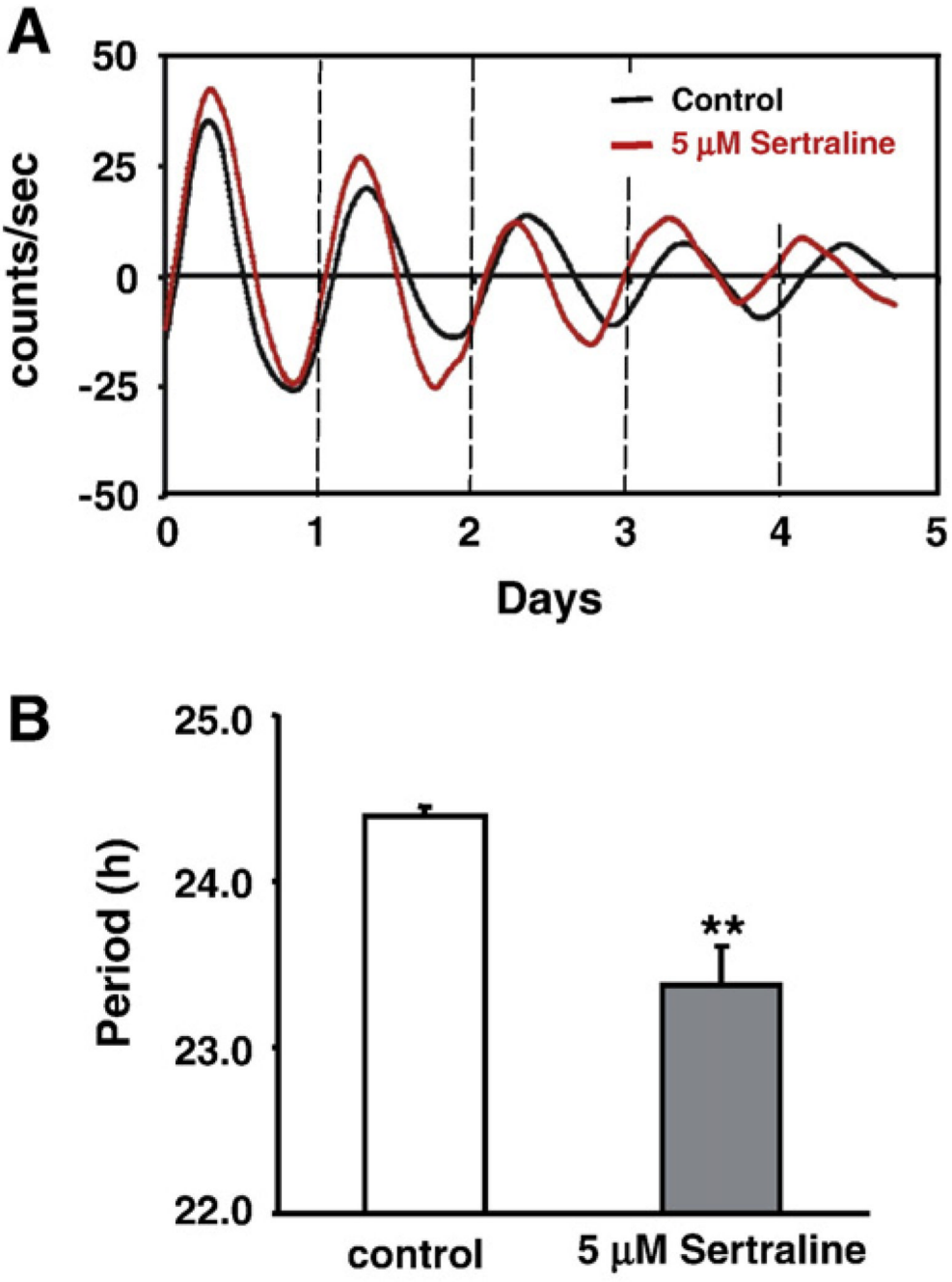

Fig. 3.

The effects of sertraline in the *Period1-luciferase* transgenic mouse SCN explants. (A) SCN slices from *Period1-Luciferase* transgenic mice showed the shortened period in the presence of 5 μM sertraline. A representative patterns for control (black) and sertraline-treated (red) samples are shown. (B) Quantitative data showing that sertraline significantly shortened the period in the SCN. Values are means±SEM ($N = 4$, from three independent experiments). Comparisons with the control condition; **, $P<0.005$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

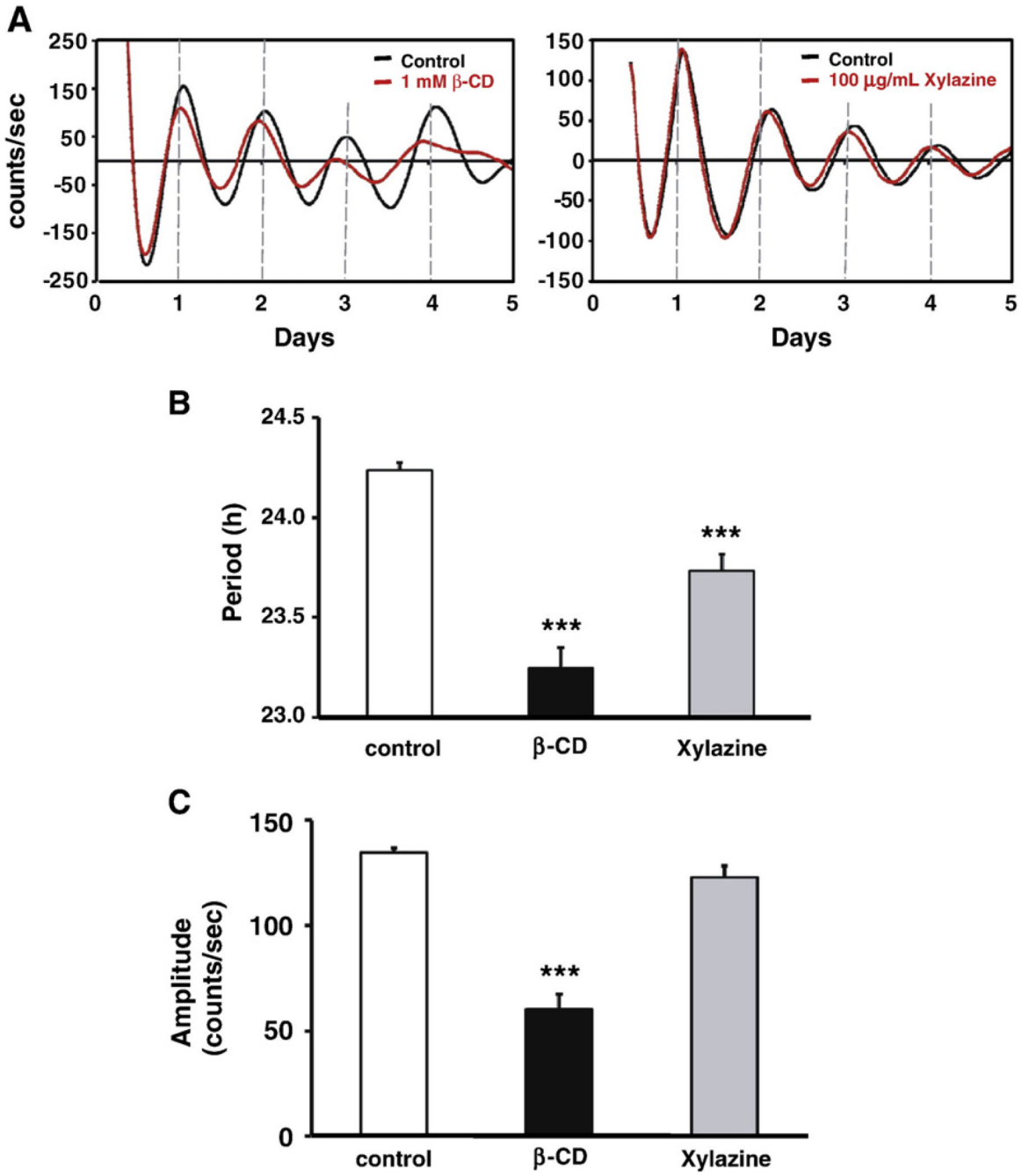

Fig. 4.

The effects of lipid raft inhibitions on the *Period1* promoter-driven bioluminescence rhythms in rat-1 fibroblasts. (A) A cholesterol chelating reagent β-CD and lipid binding reagent xylazine significantly shortened the period. Normalized data for control (black), β-CD (red in the left panel), and xylazine (red in the right panel) are shown. Amplitude was also affected by β-CD treatment. (B) Quantitative data showing the effects of β-CD and xylazine on the period. (C). Quantitative data indicate that β-CD, but not xylazine affected amplitude. Values are means ±SEM ($N = 5$, from two independent experiments. Comparisons with the control condition; ***, $P<0.005$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

The effects of selective serotonin reuptake inhibitors on circadian parameters in rat-1 fibroblasts

Chemical	Tested concentrations	Period (h)	Amplitude (counts/s)	Damping rate (days)
Control	–	24.20±0.08	135.84±3.72	2.23±0.12
DMSO	0.1%	24.21±0.05 ns	121.86±7.82 ns	2.20±0.27 ns
Citalopram	30 μM	23.29±0.17$^{**,+++}$	139.42±23.06 ns, ns	1.91±0.34 ns, ns
Fluoxetine	30 μM	23.33±0.10$^{***,+++}$	134.52±5.30 ns, ns	0.99±0.02$^{***,++}$
Fluvoxamine	30 μM	23.54±0.23$^{*,++}$	120.71±16.46 ns, ns	1.27±0.08$^{***,++}$
Paroxetine	30 μM	22.61±0.11$^{***,+++}$	130.46±16.39 ns, ns	1.38±0.17$^{**,++}$
Sertraline	30 μM	23.00±0.26$^{**,+++}$	112.89±5.38**, ns	1.05±0.11$^{***,++}$

Student τ was performed. ns, not significant;

$^{*}P<0.05$,

$^{**}P<0.005$,

$^{***}P<0.0005$ (Control vs);

$^{++}P<0.005$,

$^{+++}P<0.0005$ (DMSO vs);

ns, not significant.