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RESEARCH ARTICLE

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Small-molecule CEM3 strengthens single-cell oscillators in the suprachiasmatic nucleus

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Abstract

A robust endogenous clock is required for proper function of many physiological processes. The suprachiasmatic nucleus (SCN) constitutes our central circadian clock and allows us to adapt to daily changes in the environment. Aging can cause a decline in the amplitude of circadian rhythms in SCN and peripheral clocks, which contributes to increased risk of several chronic diseases. Strengthening clock function would therefore be an effective strategy to improve health. A high-throughput chemical screening has identified clock-enhancing molecule 3 (CEM3) as small molecule that increases circadian rhythm amplitude in cell lines and SCN explants. It is, however, currently not known whether CEM3 acts by enhancing the amplitude of individual single-cell oscillators or by enhancing synchrony among neurons. In view of CEM3's potential, it is of evident importance to clarify the mode of action of CEM3. Here, we investigated the effects of CEM3 on single-cell PERIOD2::LUCIFERASE rhythms in mouse SCN explants. CEM3 increased the amplitude in approximately 80%–90% of the individual cells in the SCN without disrupting the phase and/or period of their rhythms. Noticeably, CEM3's effect on amplitude is independent of the cell's initial amplitude. These findings make CEM3 a potential therapeutic candidate to restore compromised amplitude in circadian rhythms and will boost the development of other molecular approaches to improve health.

KEYWORDS
CEM3, PER2, rhythm amplitude, small molecule, suprachiasmatic nucleus, synchrony

Abbreviations: ANOVA, analysis of variance; BMAL1, brain and muscle arnt-like protein-1; CEM, clock-enhancing molecule; DMSO, dimethyl sulfoxide; LUC, luciferase; PER2, PERIOD2; ROI, region of interest; SCN, suprachiasmatic nucleus; TTFL, transcriptional/translational feedback loop; ZT, Zeitgeber time.

Jos H. T. Rohling and Johanna H. Meijer contributed equally to this work.

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1 | INTRODUCTION

Most organisms on earth have developed an endogenous clock with a period of approximately 24 h, which enables them to adapt to daily changes in the environment.[1,2] The clock is located in the suprachiasmatic nucleus (SCN) within the hypothalamus and consists of approximately 20 000 neurons.[3] Malfunctioning of our clock can lead to numerous diseases, such as psychiatric disorders, neurodegenerative diseases, cardiovascular dysfunction, cancer, and ultimately to premature death.[4,5] Also dampening in amplitude of circadian oscillations, as is known to naturally occur with aging, is related to an increased risk of several diseases and weakening of clock-controlled processes.[6,7] Strengthening our clock is therefore an effective strategy to increase health.

One route to achieve this is by providing lifestyle recommendations which, unfortunately, have notoriously low compliance, and are difficult to bring into practice for the elderly as well as for patients.[8] Another potential intervention would be to strengthen the clock with drugs that are designed to enhance the amplitude of the clock. High-throughput chemical screenings have identified so-called clock-enhancing molecules (CEMs): synthetic small molecules that can alter phase, period, and/or amplitude of circadian oscillations.[9–12] One of these CEMs is CEM3 (previously reported as compound 10), which is a benzimidazole compound. CEM3 has been shown to have amplitude-enhancing effects when applied to cultured SCN explants of *Clock* mutant mice.[9] This makes CEM3 a potential therapeutic candidate to improve clock function.

Generation of circadian rhythms in the SCN occurs autonomously in all individual neurons,[13,14] and all these oscillators are synchronized in phase to produce a coherent rhythm as output of the SCN.[15] Therefore, one possibility is that CEM3 amplifies the rhythm amplitude of individual neurons. The rhythm generation is controlled by negative transcriptional–translational feedback loops at the molecular level.[16,17] Potential interference at any level of this feedback loop can lead to an increment in single-cell rhythm strength. Alternatively, CEM3 may enhance synchronization among the single-cell oscillators, as enhanced synchrony of single-cell oscillators results in increased rhythm amplitude at the network level of the SCN. In other words, at the network level, an increase in amplitude can be the result of both mechanisms.

We used bioluminescence imaging of single-cell PERIOD2::LUCIFERASE (PER2::LUC) gene expression rhythms of the mouse SCN ex vivo to evaluate the amplitude-enhancing effects of CEM3. CEM3 was administered to the SCN and single-cell rhythms, as well as their mutual synchronization, were compared before and after treatment. Our results revealed that CEM3 operates at the cellular level to enhance the amplitude of the circadian PER2 rhythm, without disrupting the phase and period of the rhythm. The finding that CEM3 operates at the cellular level will guide the search strategy for possible cellular targets of CEM3 and boost the development of molecular approaches to improve health.

2 | METHODS

2.1 | Animals

For this study, we used male homozygous PER2::LUC knock-in mice ($n = 30$), which were bred at the Leiden University Medical Center animal facility.[18] All mice (3–6 months old) were housed in groups in climate-controlled cabinets with access to food and water ad libitum. The mice were subjected to a 12:12 h light–dark cycle prior to the experiment. The experiments were performed in accordance with the Dutch law on animal welfare and have been approved by the animal experiments committee Leiden.

2.2 | Materials

CEM3 (LUF7206) was prepared in-house. The synthetic procedures largely followed the protocols described by Ndakala et al.[19] We provide the detailed corresponding protocol in Appendix S1.

2.3 | Bioluminescence imaging

Organotypic explants of the SCN were prepared as described previously.[18] In short, at Zeitgeber time (ZT) 7, in which ZT12 corresponds to the time of lights-off, animals were sacrificed (Figure S1). The brain was dissected and placed in ice-cold modified artificial cerebrospinal fluid containing (in mM): 116.4 NaCl, 5.4 KCl, 1.0 NaH_2PO_4, 0.8 $MgSO_4$, 1.0 $CaCl_2$, 4.0 $MgCl_2$, 23.8 $NaHCO_3$, 15.1 D-glucose, and 5 mg/L gentamicin (Sigma-Aldrich, Munich, Germany) saturated with 95% O_2–5% CO_2 and buffered to pH 7.4. Hypothalamic slices (200 µm thick; one per animal) containing the central SCN were made with a VT 1000S vibrating microtome (Leica, Microsystems, Wetzlar, Germany). The SCN was isolated and the explant was placed on a Millicell membrane insert (PICMORG50, Merck-Milipore, Burlington, MA) in a 35 mm petri dish. The petri dish contained medium consisting of: 1.2 mL Dulbecco's modified Eagle's medium (DMEM, D7777,

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Sigma-Aldrich) supplemented with 10 mM HEPES buffer (Sigma-Aldrich), 2% B-27 (Gibco, Landsmeer, the Netherlands), 5 U/mL penicillin, 5 µg/mL streptomycin (0.1% penicillin–streptomycin; Sigma-Aldrich), and 0.2 mM D-luciferine sodium salt (Promega, Leiden, the Netherlands). Membrane inserts containing the explants were mounted in a custom-made perfusion/imaging chamber with tubing connected to syringes allowing a push–pull application of solutions during the experiment.

The petri dish was immediately transferred to a temperature-controlled (37°C) and light-tight microscope enclosure box (Life Imaging Services, Reinach, Switzerland). The box contained an upright microscope (BX51WIF, Olympus), a cooled CCD camera (ORCA–UU-BT-1024, Hamamatsu Photonics Europe, Herrsching am Ammersee, Germany), a motorized stage (XY-shifting table 240, Luigs & Neumann, Ratingen, Germany), and focus control (MA-42Z, Märzhäuser, Wetzlar, Germany). Bioluminescence images from the SCN cultures were collected using an exposure time of 29 min, with a 1-h sampling interval. Image acquisition was controlled by Image Pro Plus software (MediaCybernetics, Warrendale PA, USA; StagePro plug-in, Objective Imaging, Cambridge, UK).

2.4 | Drug administration

In the first set of experiments, CEM3 or DMSO was administered once to the SCN in the perfusion chamber on the second day ex vivo at ZT4 (for timeline, see Figure S1). This is the same administration time as Chen et al[9] previously used. DMSO administration served as control condition. In the control conditions, the syringes contained 4.8 mL of DMEM medium supplemented with 0.1% dimethyl sulfoxide (DMSO, Sigma-Aldrich, $n = 10$). In the experimental conditions, 30 µM CEM3 (prepared in-house, $n = 8$) dissolved in DMSO was added to DMEM medium to reach the same final concentration of 0.1% DMSO. Since the amplitude enhancement effect was not sustained after the one-time application, we tested the effect of repeated applications on the amplitude of PER2 rhythms in a second set of experiments, where the drug or control solution was applied on three consecutive cycles (2nd, 3rd, and 4th; CEM3, $n = 6$; DMSO, $n = 6$). CEM3 was washed out when the next dose was administered to maintain a constant concentration of the drug in the petri dish.

2.5 | Bioluminescence analysis

A custom-made MATLAB-based (Mathworks, Natick, MA, USA) algorithm was used to analyze the time series of bioluminescence images. This algorithm has previously been described in Buijink et al.[18] Briefly, a map was created from pixels containing the SCN, and the highest luminescence intensity was calculated for each pixel within the map. In the peak intensity map, groups of 3–9 surrounding pixels with luminescence intensity above the noise level were defined as regions of interest (ROIs). Each cell-like ROI represents a "single-cell". Because of cell size, we infer that the majority of recorded cells are neurons (7–9 µm) rather than astrocytes (10–20 µm).[20] However, we cannot fully exclude the presence of other cell types and therefore we will refer to the ROIs as SCN cells. The average luminescence intensity of the pixels within a ROI was calculated for each image in the time series, which resulted in the bioluminescence trace representing PER2::LUC expression of a single cell. The raw PER2::LUC expression traces were smoothed and resampled to 1 data point per minute for the analysis of rhythm characteristics, such as peak time and period. Only single-cell traces containing at least three cycles with a period length between 20 and 28 h were included for further analysis. On average 216 single-cell traces were included within a recording (range: 98–371 single-cell traces).

To analyze the synchrony among the cells ($j = 1 \ldots N$), within an explant, the Kuramoto order parameter was calculated for the specified cycle ex vivo:

$$r = \left| \frac{\sum_{j=1}^{N} e^{i\theta_j}}{N} \right|$$

Here, θ_j represents the relative phase of the j^{th} cell. The synchronization measure can take values between 0 and 1, with 0 meaning that the phases of the cells are completely randomly distributed and 1 being fully synchronized.

To determine the amplitude changes in the PER2 rhythm, the amplitude of the rhythm before and after administration of the drug was compared. We always used the amplitude measured at both the upward and downward slope of the first cycle as baseline amplitude (Figure 1) because the upward and downward slope can differ in amplitude. To assess the amplitude changes in the rhythm at the population level, we first took the average luminescence of all single-cell time traces within an explant and then divided the amplitude of the specified cycle after treatment by the baseline amplitude. To assess the changes in amplitude at the single-cell level, we divided the amplitude of the specified cycle after treatment of the individual cells by the baseline amplitude of the cells. Next, we calculated the fraction of cells with an increase in amplitude per explant. Here, we took the averaged amplitude induction at the population level of the explants treated with DMSO as threshold to establish whether a cell responded with an amplitude increase or decrease (see Table S1 for the threshold values for single

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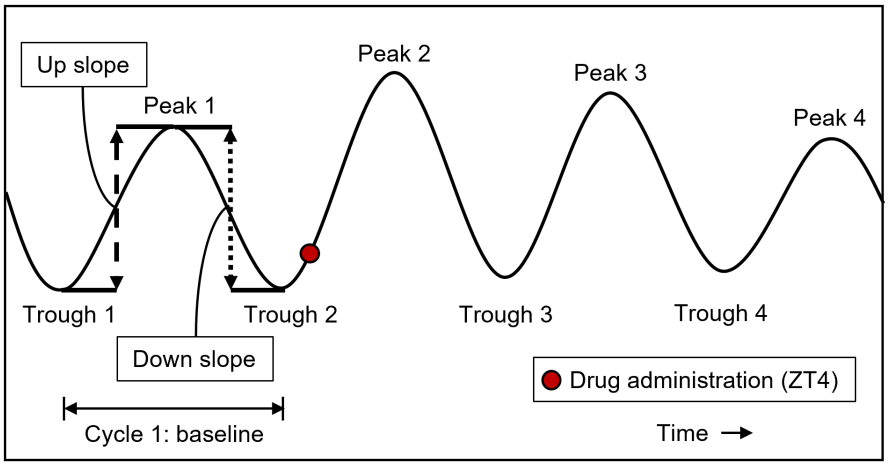

FIGURE 1 Illustration of amplitude assessment. The amplitude of the upward slope (dashed arrow) is defined as the difference in luminescence intensity between the trough before the peak and the peak (e.g., Peak 1 – Trough 1), and the amplitude of the downward slope (dotted arrow) is defined as the difference in luminescence intensity between the trough after the peak and the peak (e.g., Peak 1 – Trough 2).

TABLE 1 Amplitude-enhancing effects of CEM3 after three doses of drugs.

	Upslope amplitude induction (fold)	
	CEM3 ($n=6$)	DMSO ($n=6$)
Cycle 2	1.21 ± 0.14	0.96 ± 0.11
Cycle 3	1.16 ± 0.11	0.92 ± 0.17
Cycle 4	0.65 ± 0.26	0.52 ± 0.09
Cycle 5	0.31 ± 0.12	0.32 ± 0.13

Note: The numbers indicate the average fold induction (±SD) of the rhythm amplitude for the second until the fifth cycle compared to the first cycle (baseline) for the amplitude measured over the upslope in explants treated with CEM3 or DMSO.

administration and Table 1 and Table S2 for the threshold values for multiple administration).

2.6 | Statistical analysis

Statistical analysis of the data was performed using SPSS version 25 (IBM, Armonk, NY, USA). For comparison between groups with multiple factors, analysis of variance (ANOVA) was used, followed by a Sidak's multiple comparisons correction. For remaining comparison between groups, an independent-samples *t* test was used. The correlation between variables was analyzed with the Pearson coefficient. Differences with $p < .05$ were considered significant.

3 | RESULTS

3.1 | A single dose of CEM3 does not alter phase or period of PER2 expression within the SCN

To verify whether the previously reported effects of CEM3in *Clock* mutant mice hold up in an intact clock, we investigated the effect of CEM3 in WT mice. We first analyzed the phase, period, and amplitude of PER2 expression at the tissue level, similar to Chen et al.[9] Figure 2A shows an example of the smoothed bioluminescence traces of an explant treated with CEM3 and one treated with DMSO. The averaged peak time in PER2 expression showed no significant difference between both groups before or after treatment (Figure 2B; Sidak's test, Cycle 1, CEM3: ZT 10.9 ± 0.6, $n=8$, DMSO: ZT 11.3 ± 0.7n $n=10$; n.s.; Cycle 2, CEM3: ZT 11.5 ± 0.9, DMSO: ZT 11.5 ± 0.7; n.s.). This indicates that CEM3 does not alter the phase of the PER2 rhythm. There was also no significant difference in the period of the PER2 rhythm between both groups (Figure 2C; *t* test, CEM3: 24.1 ± 0.2 h, DMSO: 24.0 ± 0.3 h; n.s.). However, CEM3 did increase the rhythm amplitude by approximately 20% directly after administration compared to baseline amplitude, whereas the explants treated with DMSO showed a decline in amplitude of approximately 20%. This yielded effectively a difference of about 40%. The amplitude-enhancing effects of CEM3 were significant for at least the first two cycles after treatment, when the amplitude fold induction was measured at the upslope of the rhythm (Figure 2D left; Sidak's test, Cycle 2, CEM3: 1.18 ± 0.17 fold, DMSO: 0.82 ± 0.18 fold; $p < .01$; Cycle 3, CEM3: 1.04 ± 0.17 fold, DMSO: 0.76 ± 0.15 fold; $p < .01$) or at the downslope (Figure 2D right; Sidak's test, Cycle 2, CEM3: 0.92 ± 0.13 fold, DMSO: 0.75 ± 0.16 fold; $p < .05$; Cycle 3, CEM3: 0.68 ± 0.15 fold, DMSO: 0.46 ± 0.15 fold; $p < .05$).

3.2 | A single dose of CEM3 enhances the amplitude of individual cells in the SCN

After having confirmed previous studies, we continued with an analysis at the single-cell level. To evaluate whether CEM3 enhanced the rhythm amplitude at the network level or the cellular level, we analyzed the synchrony

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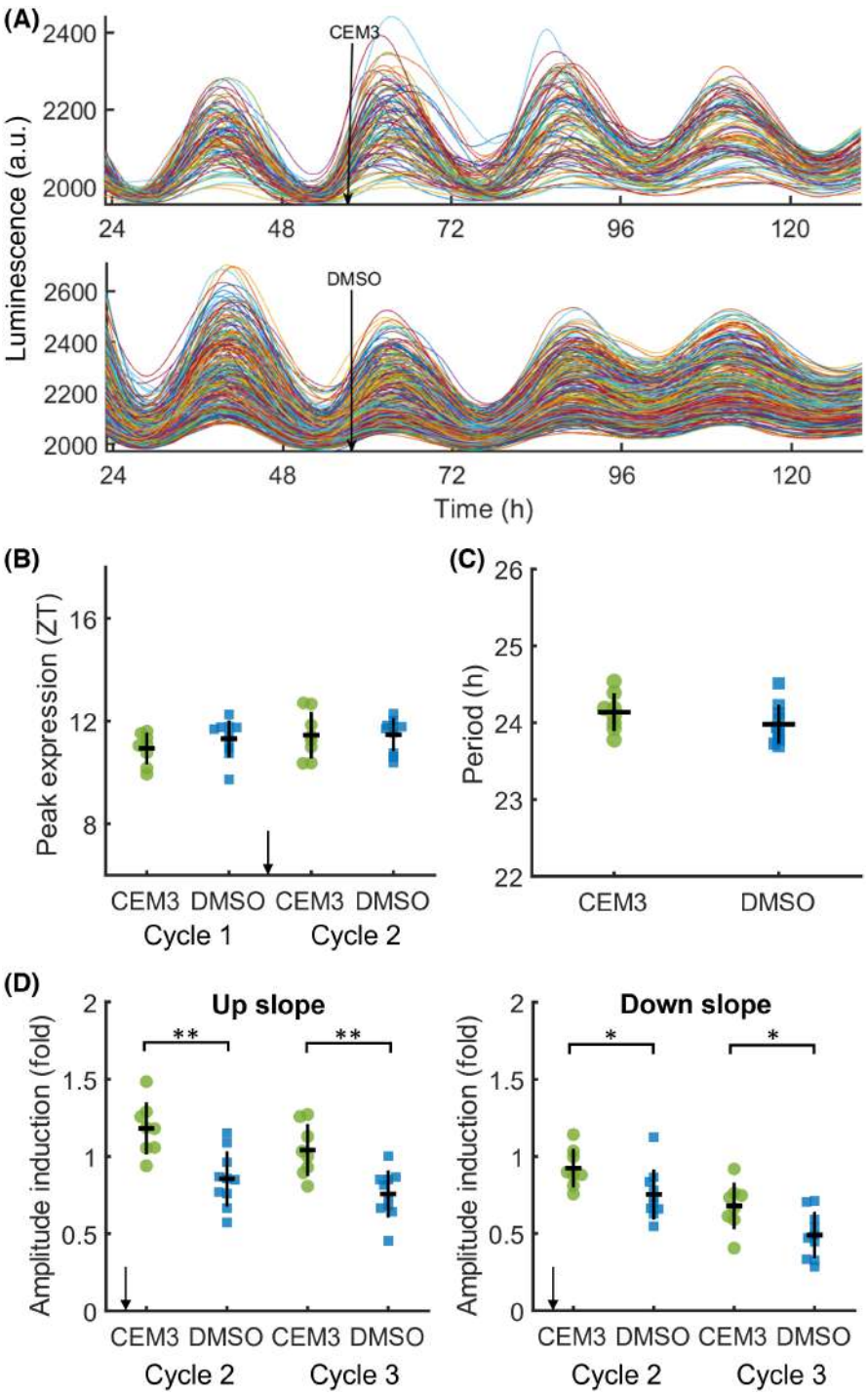

FIGURE 2 Effects of CEM3 on PER2 expression in the SCN at the population level. (A) Example of smoothed single-cell intensity traces of PER2::LUC expression from an explant treated with a single dose of CEM3 ($N = 115$ cells; top panel) or DMSO ($N = 301$ cells; bottom panel). The arrows indicate the time of drug administration. (B) Averaged peak time of PER2::LUC rhythms per explant treated with CEM3 (green circles, $n = 8$) or DMSO (blue squares, $n = 10$), plotted as Zeitgeber time (ZT) for the cycle before (cycle 1) and after (cycle 2) treatment. Black bars indicate mean ± SD. (C) Averaged period length per explant was measured over three cycles after treatment with CEM3 or DMSO. (D) Amplitude-enhancing effects of CEM3. The graphs indicate fold induction per explant of the rhythm amplitude for the second and third cycles compared to the first cycle (baseline) for the amplitude measured over the upslope (left panel) and downslope (right panel); $^{*}p < .05$, $^{**}p < .01$.

among cells in the SCN before and after administration of a single dose of drug (Figure 3A). The difference in synchronization before and after drug administration did not differ significantly between explants treated with CEM3 or DMSO (Figure 3B; *t* test, CEM3: −0.03 ± 0.02, $n = 8$, DMSO: −0.02 ± 0.05, $n = 10$; n.s.). This makes it unlikely that the increase in amplitude by CEM3 is caused by increased synchrony among cells in the SCN. Furthermore, we calculated the change in amplitude of the individual cells before and after drug administration. Figure 4A shows examples of the amplitude change in PER2 expression of individual cells from one explant. The fraction of cells showing an amplitude increase was significantly larger after treatment with CEM3 than after treatment with DMSO for two consecutive cycles, when the amplitude was measured at the upslope (Figure 4B, Sidak's test, Cycle 2, CEM3: 88.5 ± 8.1%, DMSO: 50.6 ± 18.2%; $p < .01$, Cycle 3, CEM3: 79.4 ± 12.6%, DMSO: 51.5 ± 17.5; $p < .01$). When the amplitude was measured at the downslope, there was a significant difference in the amount of cells that responded with an increase in amplitude between both groups at the second cycle, but not anymore at the third cycle (Figure 4B, right panel; Sidak's test,

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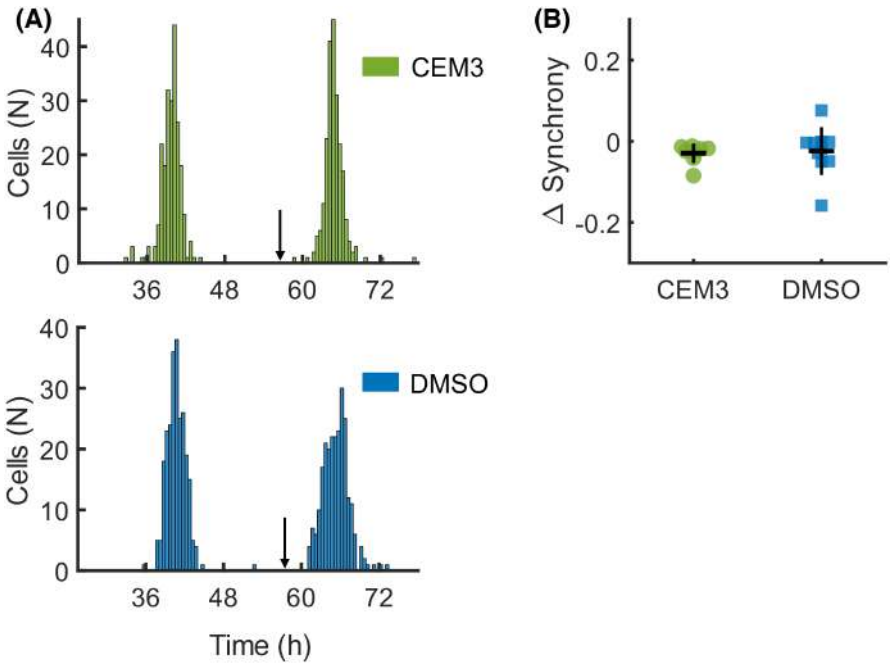

FIGURE 3 CEM3 does not alter the synchrony among cells in the SCN. (A) Representative histograms showing peak times in PER2 expression of an explant treated with CEM3 ($N = 227$ cells; top panel) or DMSO ($N = 246$ cells; bottom panel). The arrows indicate the time of drug administration. (B) Difference in synchrony as measured by the difference in Kuramoto order parameter per explant before and after treatment with CEM3 (green circles, $n = 8$) or DMSO (blue squares, $n = 10$). Positive values represent an increase in synchrony and negative values a decrease in synchrony. Black bars indicate mean ± SD.

Cycle2, CEM3: 73.4 ± 13.8%, DMSO: 43.3 ± 19.6%; $p < .01$; Cycle 3, CEM3: 75.2 ± 15.1%, DMSO: 53.7 ± 28.1%; n.s.). This could be due to the significant increase in trough luminescence intensity at the fourth trough, compared to the previous troughs, which occurred both in explants treated with CEM3 and DMSO (Figure 4C; Sidak's test, Trough 1 vs. Trough 4, $p < .01$, Trough 2 vs. Trough 4, $p < .01$, Trough 3 vs. Trough 4, $p < .01$). The trough luminescence intensity did not differ between both groups for any cycle, while there was a significant difference in peak luminescence intensity at the second and third cycles (Figure 4C; Sidak's test, Peak 2, CEM3: 2201 ± 54, DMSO: 2120 ± 53; $p < .01$; Peak 3, CEM3: 2213 ± 72, DMSO: 2132 ± 71; $p < .05$). Lastly, we investigated the relationship between the baseline amplitude of the rhythm and the change in amplitude after treatment. Scatter plots showing examples of the relationship between the baseline amplitude and the amplitude change are shown in Figure 5A for an explant treated with CEM3 and for one treated with DMSO. We found a negative relationship after DMSO treatment between the baseline amplitude and the change in amplitude, which was significantly reduced after treatment with CEM3 (Figure 5B top panel; *t* test, CEM3: −0.15 ± .017, DMSO: −0.54 ± 0.17; $p < .01$; Figure 5B bottom panel; *t* test, CEM3: −0.38 ± 0.21, DMSO: −0.66 ± 0.12; $p < .01$). This shows that CEM3 enhances the amplitude of the rhythm independent of the initial rhythm amplitude.

3.3 | Multiple doses of CEM3

To investigate the effects of administering CEM3 at three consecutive cycles, we analyzed the synchronization among cells in the SCN and we analyzed the changes in the rhythm amplitude. Here, we only show the results of amplitude measured at the upslope because the effects of CEM3 were most clearly visible at the upslope when CEM3 was administered once. The results of the amplitude values measured at downslope can be found in Figure S2 and Table S2. We analyzed the synchronization of the first five cycles ex vivo (Figure 6A). Although there is a reduction in synchrony over time, there is no significant difference in synchrony between explants treated with CEM3 or DMSO (Figure 6B; ANOVA, n.s.). Furthermore, we analyzed the change in the rhythm amplitude of the individual cells by comparing their baseline amplitude with the four cycles after the first drug administration. CEM3 increased the rhythm amplitude on average by 21% after first administration, by 16% after second administration, and surprisingly declined the rhythm amplitude by 35% after third administration compared to baseline amplitude. The full overview of the amplitude fold induction can be found in Table 1. The fraction of cells responding with an amplitude increase was significantly larger in explants treated with CEM3 than in explants treated with DMSO for the second and third cycles (Figure 7A; Sidak's test, Cycle 2, CEM3: 78.2 ± 10.9%, $n = 6$, DMSO: 48.9 ± 16.1%, $n = 6$; $p < .01$; Cycle 3, CEM3: 74.5 ± 7.4%, DMSO: 44.6 ± 24.9%; $p < .05$). However, for the amplitude at the fourth cycle, which is directly after the third dose of drugs, there was no significant difference in the fraction of cells responding with an increase in amplitude between explants treated with CEM3 or DMSO (Figure 7A; Sidak's test, Cycle 4, CEM3: 61.9 ± 24.2%, DMSO: 48.4 ± 20.1%; n.s.). For the second and third cycles, in which we found a CEM3-induced amplitude increase, it is largely the same group of cells (65.3%) that responded repeatedly with an increase

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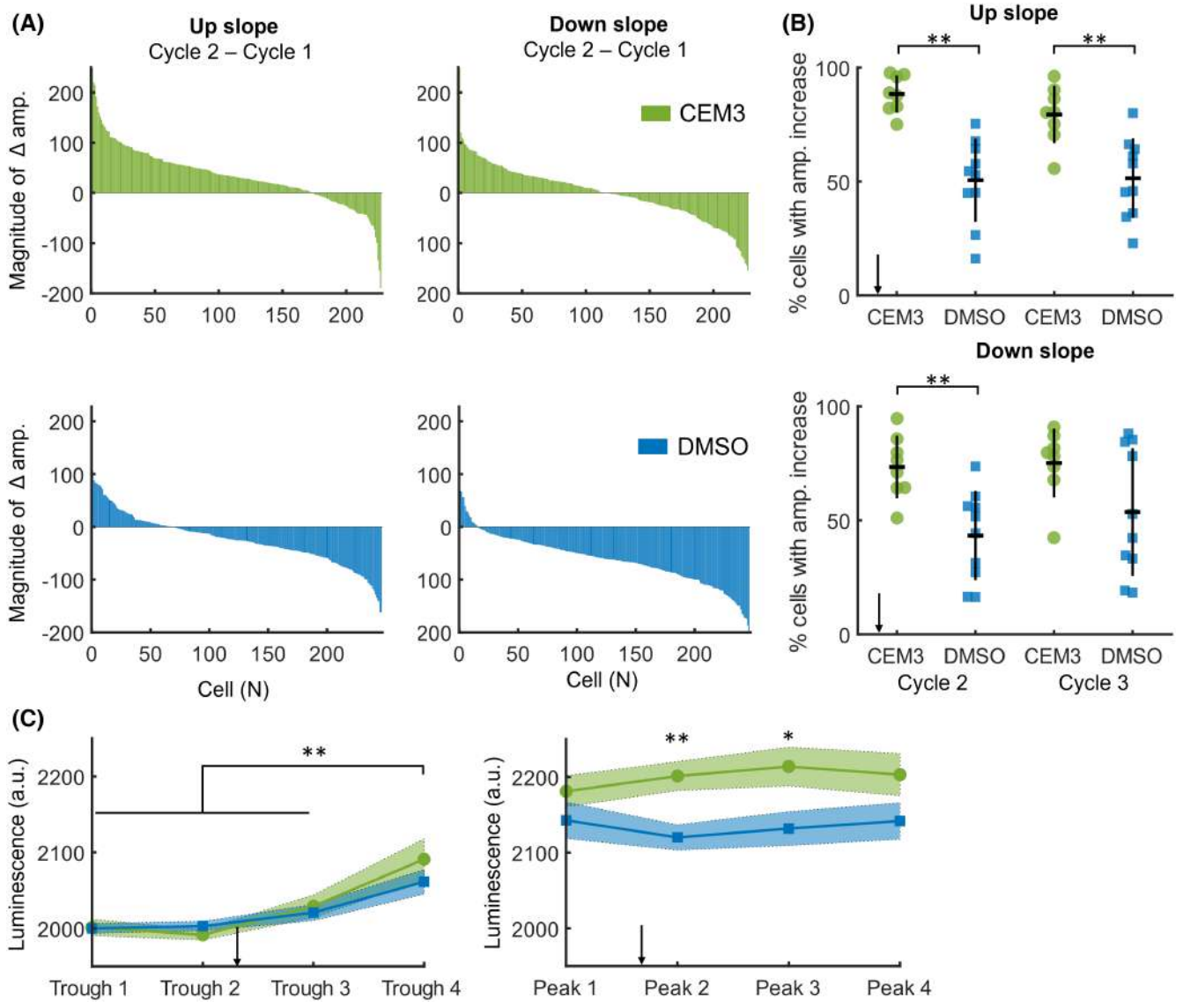

FIGURE 4 Amplitude-enhancing effects of CEM3. (A) Examples of the amplitude change in individual cells of an explant treated with CEM3 ($N = 227$ cells; top panels) or DMSO ($N = 246$ cells; bottom panels). The amplitude change is plotted in descending order with values >0 indicating an increase in amplitude and values <0 indicating a decrease in amplitude. (B) Graphs showing the proportion of cells per explant that respond with an increase in amplitude of PER2 expression after treatment with CEM3 (green circles, $n = 8$) or DMSO (blue squares, $n = 10$). The up- (left panel) and downslope (right panel) amplitude of the second and third cycles are compared with baseline amplitude. The arrows indicate the time of treatment; black bars indicate mean ± SD. (C) Graphs showing the evolution of the trough (top panel) and peak luminescence (bottom panel) as averaged over all explants treated with CEM3 (green, $n = 8$) or DMSO (blue, $n = 10$). The colored background indicates SD; $^{**}p < .01$, $^{*}p < .05$.

in amplitude (or that stayed increased) compared to its baseline (Figure 7B). To exclude the possibility that the explants are deteriorating at the fourth cycle and therefore failed to respond to CEM3, we performed additional experiments in which we only applied CEM3 at the fourth cycle ($n = 4$). Here, we found an average increase in the rhythm amplitude of 7% compared to the previous cycle (Figure S3), while in contrast a decline of 20% in amplitude was observed in controls.

In both the explants that received CEM3 once and which received CEM3 thrice, the amplitude of the rhythm was increased at the third cycle compared to baseline amplitude (i.e., cycle 1). We investigated whether the second CEM3 administration had any additional affects over the residual effects of single administration. To examine this, we took the amplitude at the second cycle (i.e., after first CEM3 administration) as new baseline amplitude, instead of the amplitude of the first cycle. When we then compared the amplitude at the third cycle with the new baseline amplitude, the fraction of cells responding with an increase in amplitude was significantly larger in explants that received CEM3 a second time than in explants that

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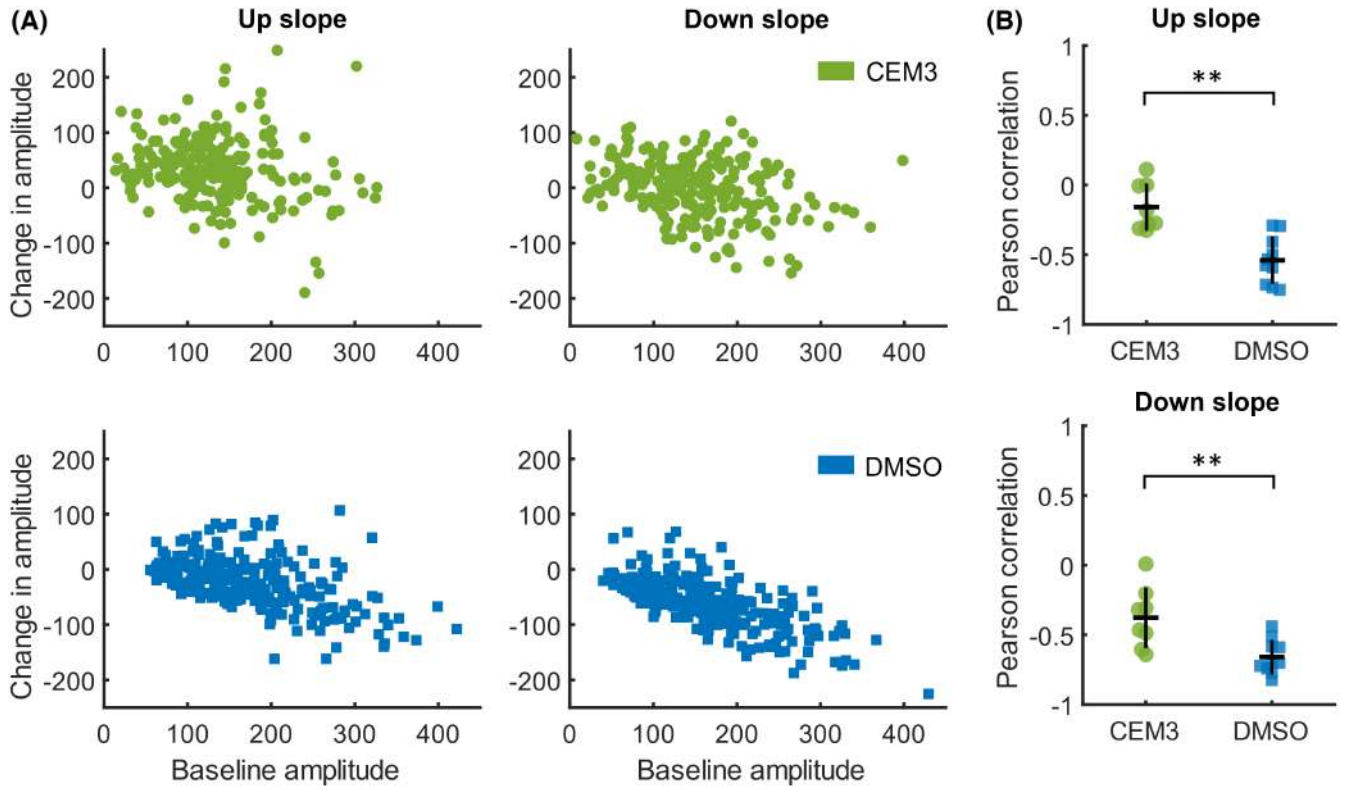

FIGURE 5 CEM3 enhances amplitude independent of the initial rhythm amplitude. (A) Example of the relationship between the baseline amplitude and amplitude change for an explant treated with CEM3 ($N = 227$ cells; top panels) or DMSO ($N = 246$ cells; bottom panels). (B) Relationship between the baseline amplitude and amplitude change per explant as measured by the Pearson coefficient; black bars indicate mean ± SD. $^{**}p < .01$.

received CEM3 only once (Figure 7C; *t* test, single CEM3: 30.1 ± 13.2%, $n = 8$, multiple CEM3: 41.2 ± 10.3%, $n = 6$; $p < .05$). This indicates that the second CEM3 administration is more effective than the residual CEM3 from single administration.

4 | DISCUSSION

A robust circadian clock is required for proper function of many physiological processes. Amplitude is one of the major circadian rhythm attributes and it can characterize the strength of the clock.[21] In aging, depression, and metabolic disorders, a dampening in rhythm amplitude is common.[22] Here, we investigated the amplitude-enhancing effects of CEM3 at the cellular level by analysis of PER2::LUC rhythms of SCN explants treated with CEM3. We found that CEM3 enhances the amplitude of the individual cells in the SCN, without altering the phase or period of the single cell. The phase coherence (i.e., synchronization) among the cells in the SCN was not affected by treatment with CEM3. This makes CEM3 a potential therapeutic candidate to restore normal amplitude in circadian rhythms.

CEM3 enhanced the amplitude of PER2::LUC rhythms in approximately 80%–90% of the individual cells in the SCN of wild-type mice. To calculate these values, DMSO-treated slices were used for reference. Previously it was shown that CEM3 was able to increase the amplitude of PER2 rhythms in SCN explants of heterozygous *Clock* mutant mice at the cell population level.[9] This left the question open whether the underlying mechanism was based on enhanced cellular synchrony or amplitude enhancement at the single-cell level. Our present results show that CEM3 operates at the single-cell level to enhance circadian rhythm amplitude.

The increase in the rhythm amplitude of the cells after treatment with CEM3 can be explained by an increase in the value of the peak luminescence. However, we also found an increase in the value of the trough luminescence over time. While the increase in peak luminescence only occurred in explants treated with CEM3 and not with DMSO, the increase in trough luminescence occurred in explants treated with CEM3 as well as DMSO. Therefore, we cannot assign the increase in trough luminescence to CEM3. Because Chen et al[9] detrended their bioluminescence time traces with a first-order polynomial, it is not possible to check whether their time traces also show an increase in trough luminescence over time. By not detrending our data, we validated that CEM3 acts by enhancing the peak.

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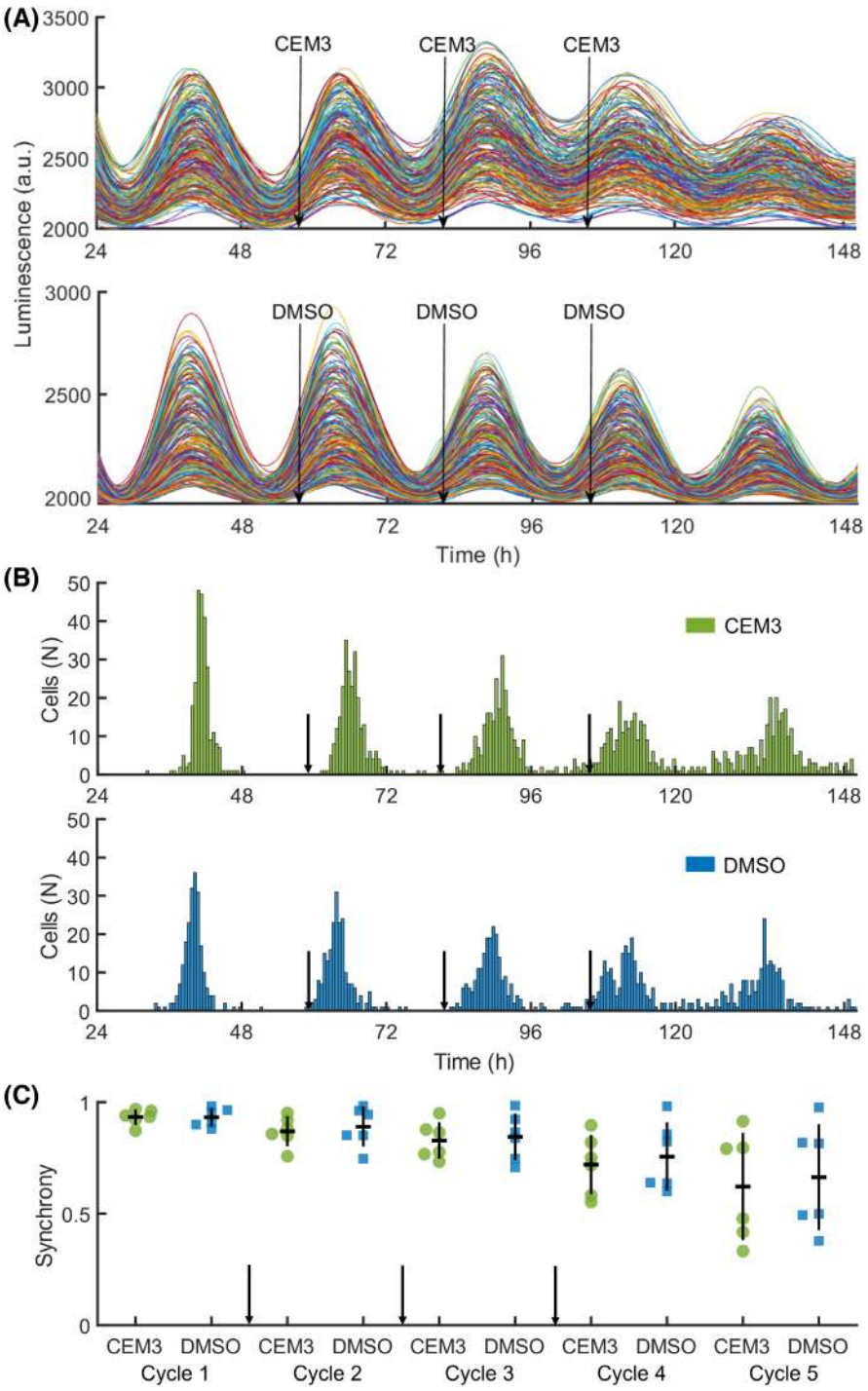

FIGURE 6 Multiple doses of CEM3 do not alter the synchrony among cells in the SCN. (A) Example of smoothed single-cell intensity traces of PER2::LUC expression from an explant treated with multiple doses of CEM3 ($N = 264$ cells; top panel) or DMSO ($N = 216$ cells; bottom panel). The arrows indicate the time of treatment. (B) Corresponding histograms with peak times of the explant treated with CEM3 (top panel) and DMSO (bottom panel). (B) Synchrony measured by the Kuramoto order parameter over time of explants treated with CEM3 (green circles, $n = 6$) or DMSO (blue squares, $n = 6$); black bars indicate mean ± SD.

Furthermore, we found that amplitude enhancement by CEM3 is independent of the initial rhythm amplitude of the cell (i.e., cells with a low initial amplitude are not more likely to gain in amplitude than cells with a high initial amplitude). In contrast, for explants treated with DMSO, we found a negative relationship between the initial rhythm amplitude and the change in amplitude at the next cycle, which is typical for normal distributed variation around the mean. Our study shows that CEM3 is capable of enhancing the rhythm amplitude in the SCN of wild-type animals. These results are nicely complementary to the study of Chen et al,[9] who showed this effect in *Clock* mutant mice. Since *Clock* mutant mice have a compromised rhythm amplitude,[23] we consider our study in WT animals important for translational purposes.

The amplitude-enhancing effects of CEM3 were largest in the first cycle after treatment. Hence, we investigated if we could maintain, or even enlarge, the CEM3-induced increase in amplitude by administering CEM3 daily. We found that the second dose of CEM3 effectively increased the rhythm amplitude on the next cycle as compared to

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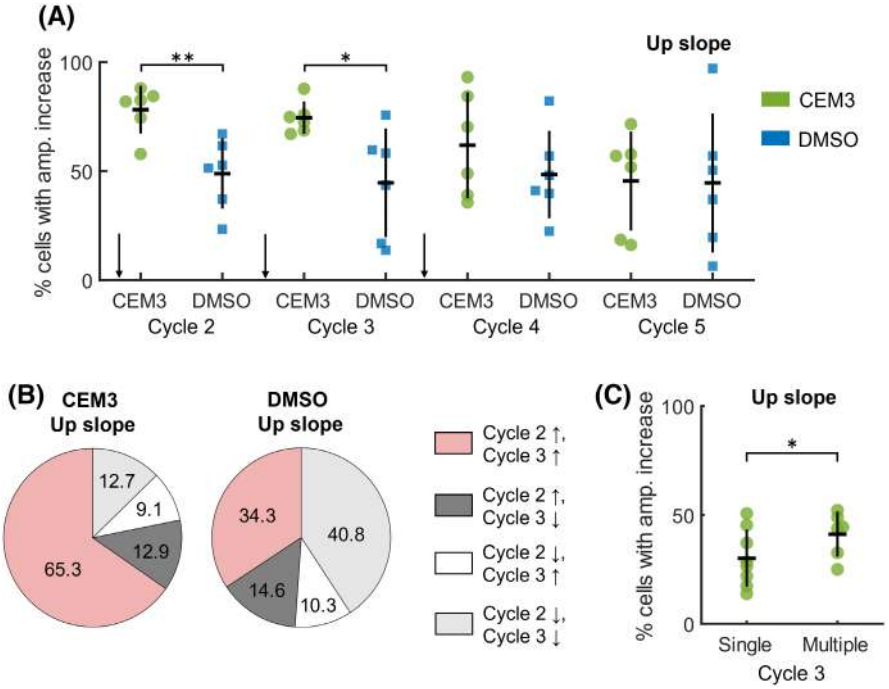

FIGURE 7 Amplitude-enhancing effects of CEM3 after multiple doses of CEM3. (A) The proportion of cells per explant that respond with an increase in amplitude of PER2 expression after treatment with CEM3 (green circles, $n = 6$) or DMSO (blue squares, $n = 6$). The upslope amplitude of the four cycles after first drug administration is compared with baseline amplitude; the arrows indicate the time of treatment and the black bars indicate mean ± SD. (B) Pie charts showing the averaged % of cells that respond repeatedly with an increase and/or decrease in amplitude for the second and third cycle compared to baseline for explants treated with CEM3 (left) or DMSO (right). (C) The proportion of cells per explant that respond with an increase in amplitude in PER2 rhythm at the third cycle, when the amplitude at the second cycle is taken as baseline amplitude. Single CEM3 administration is compared with three consecutive CEM3 administrations. Note that effects in cycle 3 are after the second administration. $^{**}p < .01$, $^{*}p < .05$.

a single dose. The third dose, however, appeared ineffective. This raised the question of whether the condition of the explant had deteriorated, prohibiting a response, or whether, alternatively, CEM3 receptors had been desensitized. Therefore, we performed an additional experiment in which we applied CEM3 on day 4 in previously untreated (i.e., naive) explants, which is the same cycle and time of application as the third dose in the experiments with multiple applications. The positive response at day 4 in previously untreated explants indicates that the explants are still viable and that receptor desensitization may have prohibited the response after repeated treatments. It remains to be tested whether receptor desensitization would also occur in vivo, in view of the continuous flow of cerebrospinal fluid.

The lack of effect of CEM3 on cell synchronization may be beneficial for use as a drug. Synchronization is important for, for instance, day-length encoding.[24] Interference with neuronal synchrony could complicate other functions of the clock. Likewise, we consider the lack of effect on period as a relevant finding. The treatment of phase-advanced or phase-delayed sleep disorders requires different treatment as compared to those who suffer from strongly attenuated rhythms, such as in aging or in depression.

There are also some other recently discovered small molecules that show clock-enhancing properties. For instance, nobiletin, a dietary flavonoid found in the peel of citrus fruits, has been shown to increase the amplitude of PER2::LUC rhythms in peripheral oscillators of heterozygous *Clock* mutant and wild-type mice.[25–27] Besides enhancing the rhythm amplitude, nobiletin also lengthens the period of the rhythm in a dose-dependent manner.[25,26] Nobilitin is expected to induce post-transcriptional enrichment of PER2, leading to an improved stoichiometric ratio of the negative arm of the transcriptional/translational feedback loop (TTFL), resulting in enhanced rhythm amplitude.[28] Because CEM3 does not lengthen the period of the oscillatory rhythm, the mechanism behind the CEM3-induced amplitude enhancement differs from nobiletin-induced amplitude enhancement.

Another small-molecule enhancing clock function is CLK8.[21] Interestingly, CLK8 increases the amplitude of brain and muscle arnt-like protein-1 (BMAL1) expression and at the same time decreases the amplitude of PER2 expression. CLK8 binds to CLOCK and disrupts the

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interaction between CLOCK and BMAL1.[21] Part of the mode of action of CLK8 is similar to nobiletin.[25] CLK8 enhances the amplitude of the circadian rhythm by stabilizing the negative arm of the feedback loop. Stabilizing the negative arm of the TTFL therefore seems the crucial factor in order to enhance circadian rhythm amplitude. However, this stabilization can be achieved in different ways. In future research, clock genes other than PER2 should be studied after administration of CEM3 to explore CEM3's mode of action further.

The target receptor of CEM3 is still unknown, representing a compelling research question. The stoichiometric relationship between the negative and positive complexes of the TTFL determines the amplitude of the rhythm.[28] It is likely that CEM3 stabilizes the negative arm of the TTFL in order to lead to amplitude enhancement of PER2. From our current data, we reason that we need to change at least two parameters in a simple oscillator model to mimic the observed effects of CEM3, as otherwise an increase in amplitude is inherently intertwined with an increase in period. In other words, both velocity and threshold need to increase in order to enhance amplitude but preserve period.

The amount, localization, and activity of clock-related proteins require precise regulation for the generation of robust and reliable circadian rhythms. If an organism cannot adapt adequately to environmental changes, due to dampened circadian rhythms, this would not only defeat the evolutionary purpose of the circadian clock but also compromise the clock's significance for human health. Here, we shed light on the potential of a small molecule, CEM3, to enhance circadian rhythm amplitude. Evidently, CEM3 enhances the amplitude of both healthy and dampened single-cell oscillators. CEM3 may be used as a starting point for the development of future therapeutics to restore low amplitude associated with aging, metabolic syndrome, and mood disorders.

AUTHOR CONTRIBUTIONS

Johanna H. Meijer, Adriaan P. IJzerman, Stephan Michel, Jos H. T. Rohling, and Anouk W. van Beurden designed the study. Jaco D. P. van Veldhoven and Adriaan P. IJzerman synthesized CEM3 for the experiments. Mayke M. H. Tersteeg, Anouk W. van Beurden, and Stephan Michel performed the experiments. Anouk W. van Beurden and Jos H. T. Rohling analyzed the data. Anouk W. van Beurden, Jos H. T. Rohling, and Johanna H. Meijer wrote the paper with input from all other authors, and all authors reviewed and approved the paper.

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DISCLOSURES

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the methods and/or supplementary material of this article.

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SUPPORTING INFORMATION

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