Hypo-Osmotic Stress Impact on Cell Circadian Rhythms

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Abstract

Brain and muscle ARNT-like protein 1 (BMAL1) is a key positive regulatory element in the transcription-translation negative feedback loop (TTFL) responsible for circadian rhythms. BMAL1 is the only single clock gene deletion that results in the complete removal of all rhythms. Previous research indicates that hypo-osmotic stress causes caveolae deformation which in turn disrupts the cell circadian rhythm though the exact mechanism is not known. A follow-up experiment showed that hypo-osmotic stress disrupts cell growth and the TTFL through cytosolic localization of BMAL1. To further explore the effects of hypo-osmotic stress on the cell cycle and circadian rhythm pathway, BMAL1 localization was compared in undifferentiated PC12 cells under both osmotic stress and control conditions. PC12 cells were chosen due to their lack of caveolae. Additionally, the impact of hypo-osmotic stress on cell growth and its potential use for cell synchronization was tested using FUCCI cell growth stage fluorescence. The PC12 experiment indicated that cells not containing caveolae maintain their circadian rhythm cycle even when osmotically stressed, further supporting previous theories connecting caveolae deformation to circadian rhythm disruption. The FUCCI experiment indicated that osmotic stress is not effective for cell synchronization.

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Introduction

Circadian rhythms are the biological clocks within bodily systems that temporally regulate many of an organism's daily functions including sleep, hunger, and even temperature (Qifti, 2022). Cellular circadian rhythms function on a set ~24-hour cycle regulated by the production, dimerization, and decay of specific proteins in what is known as the transcription/translation feedback loop (TTFL). This feedback loop manages the transcription factors that activate the circadian rhythm genes, namely the Period (PER) and Cryptochrome (CRY) genes. Gene expression oscillates over 24 hours causing the protein products to build up over half of a full 24-hour day and then lessen in the other half (Figure 1). The build-up of these proteins is ultimately what inhibits their own production until protein turnover allows expression to restart creating a self-regulating negative feedback loop. The basic loop follows PER and CRY proteins' inhibition of the transcriptional activator complex CLOCK/BMAL1 (Andreani et al., 2015). The genes encoding for the proteins involved in this cycle are known as "clock" genes. Though there are many other clock genes, the key players in the primary and secondary feedback loops are brain and muscle ARNT-Like 1 (BMAL1), CLOCK (from Circadian Locomotor Output Cycles Kaput), PER, CRY, REV-ERB, and retinoic acid receptor-related orphan receptor (ROR) (Solt et al., 2011).

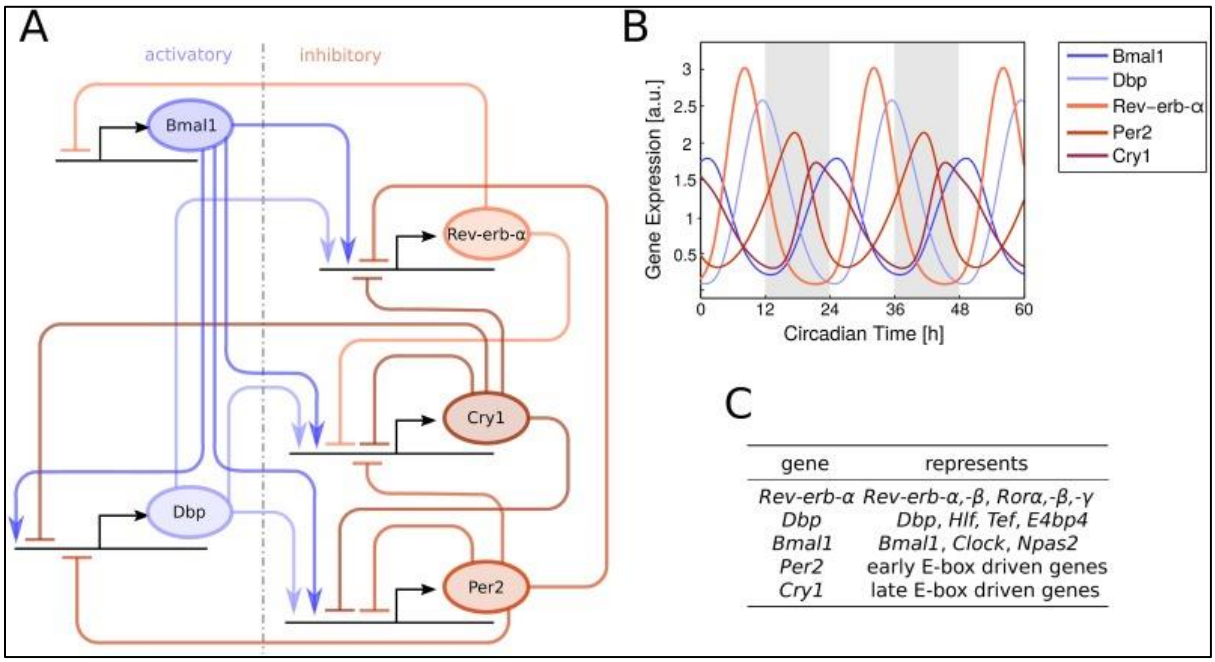

Figure 1. Circadian Oscillator Model represented by a network graph and gene expression oscillation graph with a key. (A) Network graph of the circadian oscillator model. Activating and inhibiting factors and connections between genes are colored in blue and red, respectively. (B) Simulation of gene expression over time of circadian oscillator model genes. (C) Key showing that each "gene" in the model represents a group of genes with similar functional characteristics. (Pett et al., 2016)

The TTFL includes positive regulatory elements promoting gene expression and protein products inhibiting gene expression (Hurley et al., 2016). Positive regulatory elements bind to a clock gene promoter resulting in gene expression. The two key positive regulatory elements are CLOCK and BMAL1 which together form a dimeric transcription factor activating PER, CRY, REV-ERB, and ROR protein expression. In the primary feedback loop, CLOCK/BMAL1 binds to the E-box upstream of PER and CRY, promoting their transcription (Figure 2). Once enough protein products accumulate in the cytoplasm, they are transported into the nucleus where they inhibit the transcriptional activity of the CLOCK-BMAL1 dimer, repressing their own expression. The clock genes are consequently transcribed at low levels until the CRY and PER protein products are degraded, allowing for CLOCK/BMAL1 to bind to the promoter and restart transcription (Qifti, 2022). The proteins also regulate downstream genes that are not part of the feedback loop causing daily rhythms in other processes, such as metabolism (Hastings et al., 2008). The secondary loop of the TTFL includes ROR and REV-ERB which regulate the expression of BMAL1. REV-ERB transcription is activated by BMAL1/CLOCK and repressed by CRY/PER accumulation. In turn, REV-ERB represses BMAL1 transcription. ROR, an activating element, competes with REV-ERB for binding of their shared DNA binding elements, the RRE, in the BMAL1 promoter (Figure 2). The oscillating expression of ROR and REV-ERB leads to the circadian pattern of BMAL1 expression. This secondary feedback loop interconnects the positive and negative regulatory arms of the primary circadian loop (Solt et al., 2011).

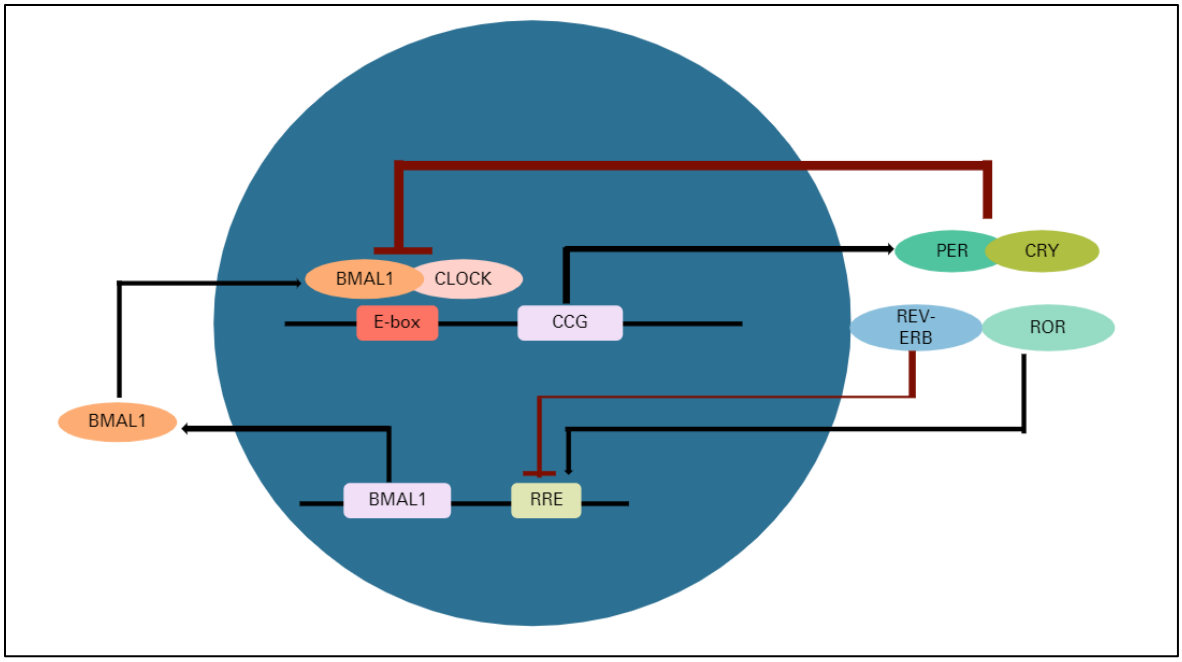

Figure 2. Model of localization and regulatory impacts of clock proteins in the nucleus (blue) and the cytoplasm. BMAL1/CLOCK dimer binds to the E-box causing the expression of clock-controlled genes (CCG) which accumulate in the cytoplasm. PER and CRY proteins inhibit the activity of BMAL1/CLOCK. REV-ERB competes with ROR to bind to RRE. REV-ERB inhibits expression of BMAL1 while ROR promotes expression of BMAL1. BMAL1 protein travels from the cytoplasm to the nucleus to enter the cycle.

The circadian cycle, spanning 24 hours, includes characteristic delays between mRNA accumulation, protein accumulation, and gene suppression due to translation dynamics, post-translational protein modification, protein dimerization, and protein localization to the nucleus (Hastings et al., 2008). The cycle's period remains consistent in each individual and is typically around 24 hours (Figure 1). The TTFL is a closed loop that will return to its oscillatory path on its fixed 24-hour period even if it is disturbed. Though the circadian rhythm cycle is self-regulating, signals and stressors from the environment can have an effect. For instance, exposure to light at a different time of day can impact when the body turns on PER and CRY gene expression (Bedrosian & Nelson, 2017). Previous research conducted in the Scarlata lab on the impact of hypo-osmotic stress on cells, specifically regarding caveolae, found that clock genes were being affected as well as an unintended result of the osmotic stress. Caveolae are a type of lipid raft that form cup-like indents in the plasma membrane of many vertebrate cells. They are made up primarily of caveolin and cavin proteins which affect the local morphology in the cell

membrane to form caveolae, playing a role in signaling, membrane structure, and mechanical strength. Apart from organizing caveolae, cavin-1 promotes ribosomal RNA transcription in the nucleus (Qifti, 2022).

The Scarlata lab research found that, when subjected to hypo-osmotic stress, caveolae undergo deformation and cavin-1 is relocalized to the nucleus. This study used WKO smooth muscle cells to identify the genes whose expression was changed during caveolae deformation from osmotic stress and through this they found that osmotic deformation of caveolae affects the cell circadian rhythm at 12 hours, but not at 24 hours. This is believed to be caused by the relocation of Cavin-1 which impacts gene transcription. Additionally, the research found that osmotic stress induces the formation of stress granules in the cytoplasm of neuronal and smooth muscle cells. Previous studies show certain stress granule proteins to be binding partners with cytosolic BMAL1. Cytosolic BMAL1, when phosphorylated, regulates ribosomal translation in cells, associating with several translation initiation factors. From this, it was hypothesized that when subjected to 12 hours of hypo-osmotic stress, BMAL1 is phosphorylated, potentially binding to stress granule proteins, in the cytoplasm, and does not dimerize with CLOCK to initiate PER and CRY transcription. At 24 hours, despite continuing caveolae deformation, stress granule protein synthesis lowers and BMAL1 is able to return to the TTFL. Some studies relate osmotic stress with G1 cell cycle delay indicating that exposing cultured unsynchronized cells to prolonged osmotic stress may synchronize the cell cycle similar to how serum starvation is used and that BMAL1 phosphorylation may play a key role in this process (Qifti, 2022).

A follow-up study was conducted by the Scarlata lab to track BMAL1 localization in WKO cells after 12 and 24 hours of prolonged osmotic stress. To characterize the changes in cell growth induced by osmotic stress, the area of nuclei was measured to track cell growth after refeeding serum starvation synchronized cells. It was found that after serum starvation, the cell cycle is arrested in the G1 phase and that 16 hours of refeeding using either regular or osmotic media induced the S-phase while 8 hours of refeeding did not, indicating that osmotic media alone does not cause cell cycle arrest. Additionally, by fluorescently tagging BMAL1 this study found that hypo-osmotic stress conditions at 12 hours caused decreased BMAL1 intensity in the nucleus compared to regular media while at 24 hours BMAL1 intensity in the nucleus had returned to normal levels. Though this experiment did not synchronize the cells beforehand and therefore

may have had other contributing factors, the results were consistent with previous research indicating that osmotic stress disrupts the circadian rhythm cycle through phosphorylation of BMAL1 in the cytoplasm.

Not all cells have caveolin. One such cell line is undifferentiated PC12 cells which are derived from tumors in the rat adrenal medulla. PC12 cells have an embryonic origin from neuroblastic cells allowing them to easily differentiate into neuron-like cells even though they are not considered adult neurons (Greene & Tischler, 1976). Typically, PC12 cells are used for studying neuronal differentiation and other areas of neuroscience. Typically, PC12 cells do not have caveolin (Chamberlain et al., 2001) making them useful as a comparison of osmotic stress reactions to WKO smooth muscle cells which do have caveolin.

The overall objective of the following research experiment was to identify the impact of prolonged hypo-osmotic stress on cell circadian rhythms in cells not containing caveolae to determine the role of caveolae as a key factor in this response. This was accomplished by synchronizing undifferentiated PC12 cells through serum starvation and then subjecting them to various lengths of prolonged osmotic stress. BMAL1 was then fluorescently stained, and its localization analyzed. Additionally, an experiment was conducted to analyze the effectivity of osmotic stress as a means of cell synchronization. This experiment used RPE cells with Fluorescence Ubiquitin Cell Cycle Indicator (FUCCI) technology which are designed to fluoresce at different stages in cell growth (G1: red, S/G2: green, G1/S transition: both) (Zielke & Edgar, 2015) to determine cell cycle arrest and synchronization in cells subjected to prolonged osmotic stress. These cells contain caveolae and therefore would be expected to undergo BMAL1 phosphorylation and circadian rhythm arrest when subjected to hypo-osmotic stress.

Methodology

Serum Starvation Cell Synchronization

To determine the ideal length of time of serum starvation for cell cycle synchronization, PC12 cells were subjected to 8, 12, 16, and 24 hours of serum starvation. This was done by splitting stock PC12 cells into six 35 mm dishes with glass bottoms for microscopy. Cell splitting was done by first aspirating the media from the stock dish and washing the dish 3x with 2 mL PBS. The cells were collected using 3 mL complete media and a pipette to remove cells from the dish bottom. Each glass bottom dish was given 500 µL suspended cell solution. All conditions were allowed to proliferate and adhere overnight at 37℃. The complete media in each dish was aspirated before being replaced with either complete media or serum starve media lacking horse serum and FBS. The cells were not washed at this step. Four of the dishes had their media replaced with serum starve media while two control dishes were given new complete media. All dishes were returned to incubation until fixation. The starvation dishes were fixed with 500 µL 3.7% formaldehyde solution in diH_2O for 10 minutes at room temperature at 8, 12, 16, and 24 hours respectively. The control dishes were fixed at 24 hours and 48 hours respectively. All cells were washed 3x with 1 mL non-sterile (NS) PBS and refrigerated in 1 mL NS-PBS following fixation. The next step was to image each dish using a 60x confocal microscope. To determine the stage of cell growth the areas of cell nuclei were measured using ImageJ software. A graph was created to compare nuclei sizes between conditions. A one-way ANOVA test was used across the data set and between pairs of data to determine significance.

PC12 Cell BMAL1 Localization

Cell Synchronization and Osmotic Stress Conditions

Undifferentiated PC12 cells were synchronized in the G1 stage using serum starvation. After splitting stock cells into five 35 mm glass bottom dishes using the previous procedure, the cells were allowed to proliferate and adhere overnight at 37℃. The media was then aspirated and replaced in all dishes with serum starve media without washing. After 16 hours incubation, the

dishes had their media aspirated and replaced again without washing. Two dishes of cells were subjected to hypo-osmotic stress through a 1:1 ratio of diH_2O with complete media. Two dishes were given regular media for control conditions. These four dishes were incubated for set times. One dish was fixed directly after serum starvation as a 0-hour control. One osmotic stress condition dish and one control dish were fixed at 12 hours. The last two dishes were fixed at 24 hours. The fixation process included 500 µL of 3.7% formaldehyde in PBS for 30 minutes at room temperature for each dish. At 25 minutes into fixation, 5 µL of 4′,6-diamidino-2-phenylindole (DAPI) was added to each dish. To prepare the dishes for refrigeration the formaldehyde solution was removed, and each dish was washed 3x with 1 mL NS-PBS. The cells were kept in a cold room in 1 mL NS-PBS.

Immunofluorescent Cell Staining and Imaging

Immunofluorescent staining was used to stain the cells for BMAL1. First, the cells were permeabilized by washing them 3x for 10 minutes each with 2 mL buffer solution containing NS-PBS and 0.4% Triton X. This buffer was then replaced with a blocking solution made up of NS-PBS, 5% goat serum, 1% BSA, and 50 mM glycine. Each dish was given 2 mL blocking solution for 30 minutes. The primary antibody was prepared by a 1:200 dilution of BMAL1 antibody (rabbit) in NS-PBS and 1% BSA. After removing the blocking solution, 0.5 mL of the primary antibody was added to each dish and the dishes were incubated for 1 hour at room temperature. The antibody was removed, and the cells were washed 3x with 2 mL NS-PBS for 10 minutes each. The secondary antibody was prepared for 0.5 mL per dish using a 1:400 dilution of Alexa Fluor 488 (anti-rabbit) in NS-PBS. The last wash was removed, and the secondary antibody was added. The dishes were immediately placed in a larger dish wrapped in foil due to light sensitivity. This dish was incubated for 1 hour at 37℃. Finally, the cells were washed 3x again with 2 mL NS-PBS for 10 minutes each and placed in the cold room still in tin foil. To measure and compare the intensity of BMAL1 fluorescence a 60x confocal microscope with fluorescence capabilities was used. Multichannel images of cells were taken using the FITC (green) channel for BMAL1 fluorescence and the DAPI (blue) channel for the nucleus. Using ImageJ, the intensity of the BMAL1 fluorescence was measured in the nucleus, the entire cell, and outside of any cells to be subtracted as background noise. The nuclear intensity was divided by the whole-cell intensity to determine BMAL1 localization in either the nucleus or the

cytoplasm. A graph was made to compare BMAL1 localization between conditions. A one-way ANOVA test was used across the data set and between pairs of data to determine significance.

FUCCI Cell Growth Synchronization

Flow Cytometry

To determine the viability of osmotic stress as a method of cell synchronization FUCCI cells were subjected to varying lengths of hypo-osmotic stress. FUCCI cells were split from stock into five 35 mm glass bottom dishes for imaging and five 60 mm dishes for flow cytometry. Cells were allowed to proliferate and adhere overnight. Similar to the previous experiment dishes were then subjected to five different conditions, this time in duplicate: 0-hour control, 12-hour osmotic stress, 24-hour osmotic stress, 12-hour control, and 24-hour control. The 60 mm dishes were prepared for flow cytometry by fixation. These cells were transferred to microtubes by first aspirating the media from each dish and washing the dish 3x with 1 mL PBS. The cells were subjected to 4 minutes of 500 µL trypsin to release them from the dish bottom and then mixed with 1 mL media and pipetted into the centrifuge tubes. The cells were collected by centrifugation and the supernatant was discarded. The cells were then resuspended in 0.5 mL of 4% formaldehyde in NS-PBS solution and fixed for 15 minutes at room temperature. After fixing the cells were washed by centrifugation with excess PBS and resuspended in 1 mL PBS after discarding the supernatant. The five tubes of cells were then stored in a cold room until analysis. To analyze the cells, a flow cytometer was used. The tubes were put on ice and run through a flow cytometer one by one for 5 minutes each. Graphs were created based on the different expected fluorescent properties of the FUCCI cells.

Cell Staining and Imaging

The cells in the 35 mm glass bottom dishes were analyzed using fluorescent microscopy. Each dish was fixed and DAPI stained according to the procedure used with the PC12 cells except with a 4% formaldehyde solution. The cells were then imaged using a 60x confocal microscope

with fluorescence capabilities. Multichannel images of cells were taken using the FITC (green) channel for S/G2 stage fluorescence, the TRITC (red/orange) channel for G1 stage fluorescence, and the DAPI (blue) channel for the nucleus. ImageJ was used to quantify the percentage of cells in the G1 phase (red only), G1/S phases (both), and S/G2 phases (green only) for each condition. A graph was created to compare cell stage ratios between conditions. A one-way ANOVA test was used across the data set and between pairs of data to determine significance.

Results

Cell Cycle Stage Analysis by Nuclei Area

To determine cell synchronization after serum starvation, the area of the nuclei of cells subjected to varying lengths of serum starvation was measured in images taken by a 60x confocal microscope and analyzed in ImageJ software (Figure 3). At 8 hours of serum starvation, the average area of the nucleus was 38.257 μm^2. At 12 hours the dish had too much debris for data collection and analysis. At 16 hours the average area of the nucleus was 40.876 μm^2 and at 24 hours it was 35.724 μm^2. The average area for the 24-hour and 48-hour controls was 62.793 μm^2 and 58.558 μm^2 respectively. A column graph of the data with SEM-derived error bars was created using PRISM software (Figure 4). A one-way ANOVA test showed high significance ($p<0.001$) between pairs of each serum starvation condition with each control condition.

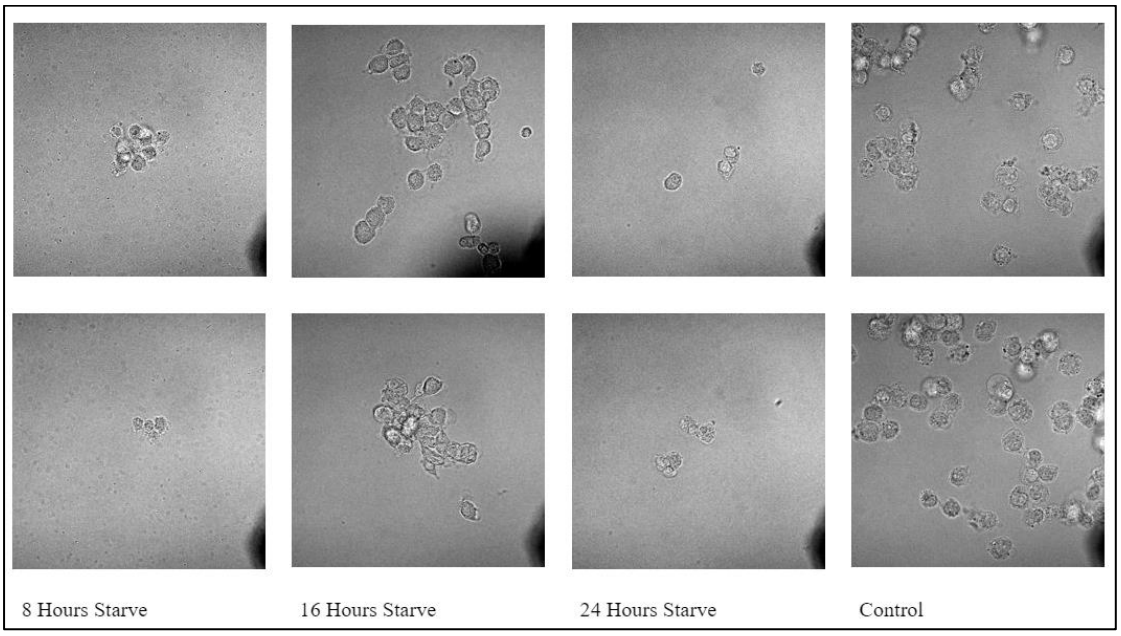

Figure 3. Images of fixed PC12 cells at different time conditions (L-R): 8-hour serum starvation, 16-hour serum starvation, 24-hour serum starvation, 24-hour control. The 8-hour and 24-hour starvation conditions show significantly lower populations of cells than the other conditions indicating slower growth and higher levels of cell death.

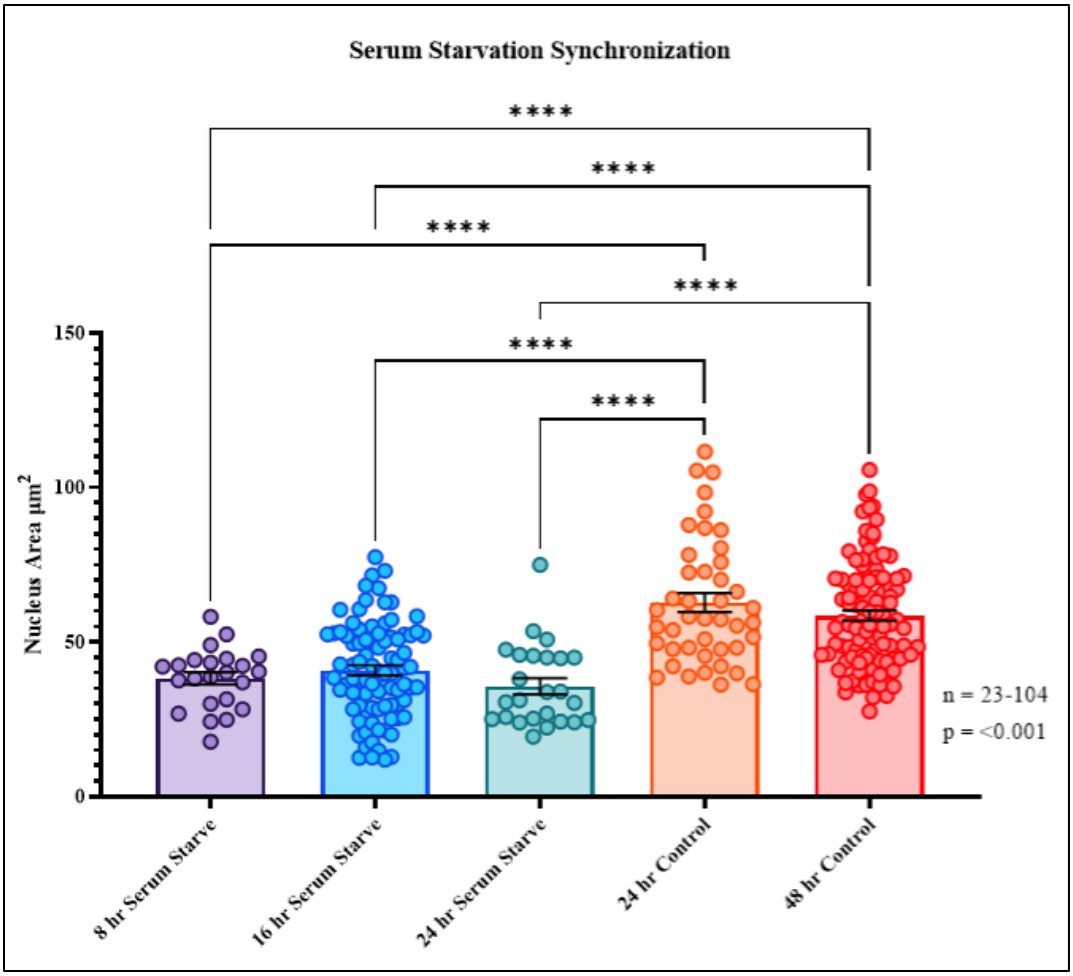

Figure 4. Column graph with individual data points and SEM error bars comparing nuclei area measurements across conditions. Y-axis shows the nuclei area in μm^2. X-axis shows the different time conditions. One-way ANOVA testing showed significance across the data and between each control and serum starvation condition.

BMAL1 Localization Analysis by Comparative Fluorescence Intensities

BMAL1 localization in cells subjected to prolonged hypo-osmotic stress was analyzed through fluorescence intensity comparisons between the nuclei and the whole cell. This was accomplished by first synchronizing the cells at 16 hours of prolonged serum starvation and then refeeding cells with either osmotic or regular media for either 0 hours, 12 hours, or 24 hours. BMAL1 was immunostained with a fluorescent tag and the nuclei were stained with DAPI (Figure 5). ImageJ was used to outline the nucleus and then in the images showing BMAL1 fluorescence find the mean intensity of fluorescence within the nuclear outline and within the

whole cell. The mean intensity of the whole cell outline transposed onto the background was subtracted from both numbers to remove background noise from the data. The average mean intensity of the nuclei divided by the mean intensity of the whole cell was 0.9154 at 12 hours of regular media refeeding, 0.9438 at 12 hours of osmotic media refeeding, 0.9849 at 24 hours of regular media refeeding, 0.9801 at 24 hours of osmotic media refeeding, and 1.05903 for the 0-hour control. A column graph of the data with SEM-derived error bars was created using PRISM software (Figure 6). A one-way ANOVA test showed high significance ($p<0.0001$) between the 0-hour control condition and all other conditions and between the 12-hour control and 24-hour osmotic stress condition. No significance was found between other lengths of refeeding times or between osmotic media and regular media conditions.

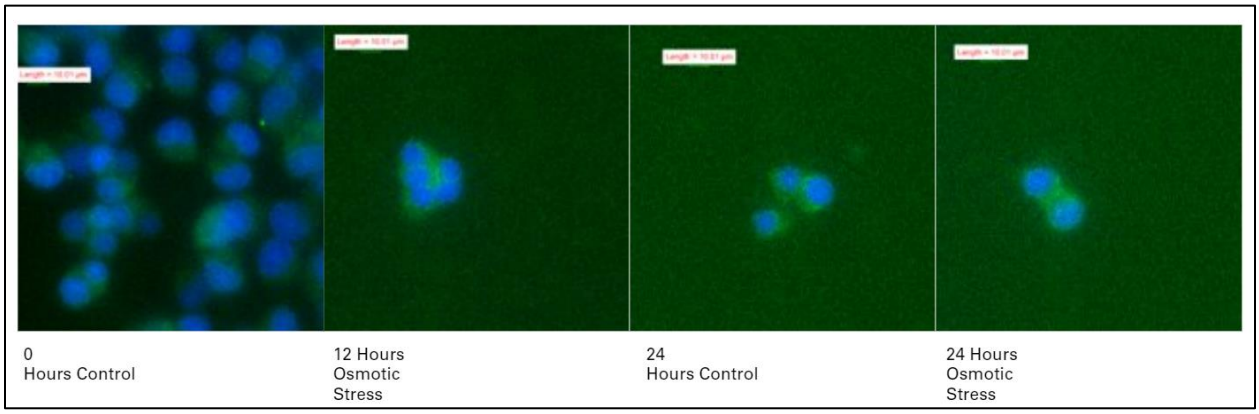

Figure 5. Images of fixed PC12 cells with DAPI and FITC (BMAL1) fluorescence at different time conditions (L-R): 0-hour control, 12-hour osmotic stress, 24-hour control, 24-hour osmotic stress. Scale bars showing 10.01 µm are included.

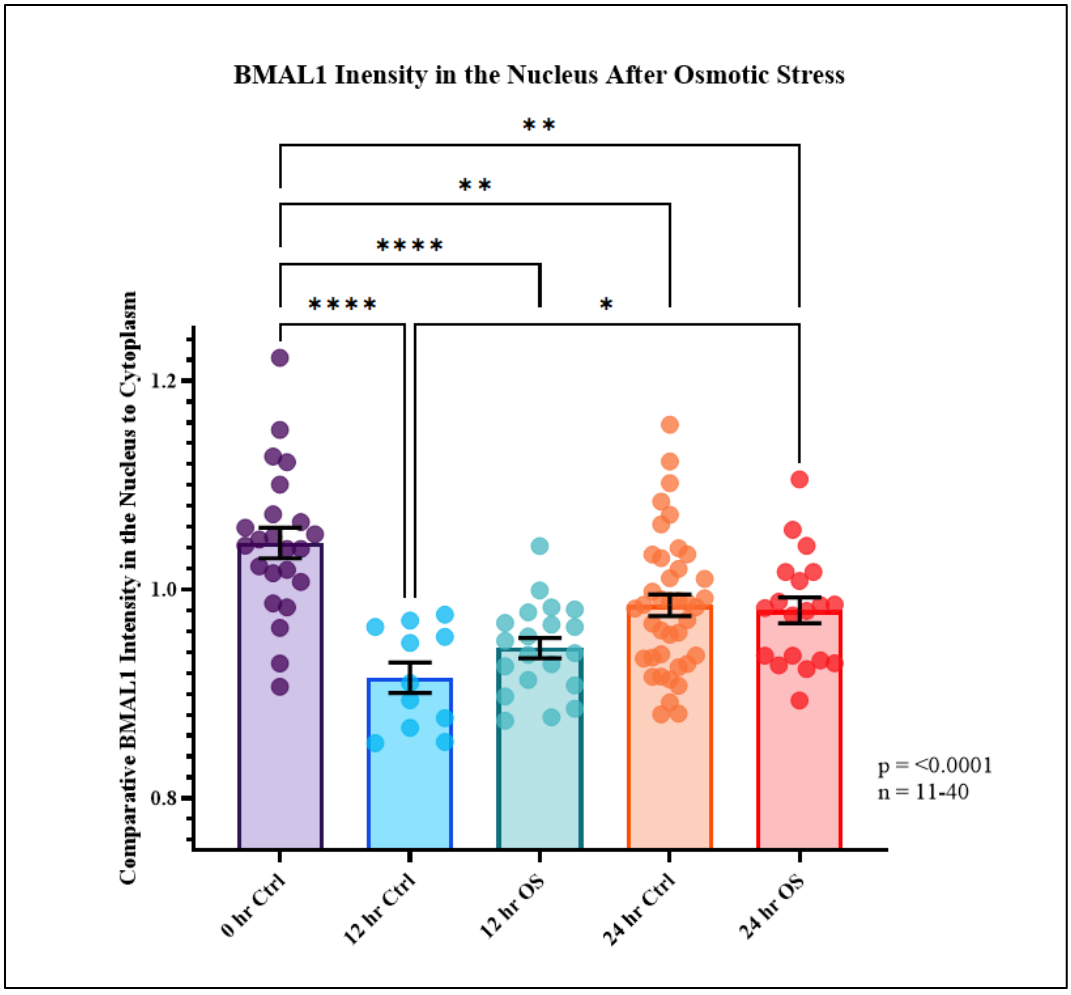

Figure 6. Column graph with individual data points and SEM error bars comparing relative BMAL1 intensity in the nucleus across conditions. Y-axis shows the mean BMAL1 intensity in the nucleus divided by the mean BMAL1 intensity in the whole cell after subtracting background noise. X-axis shows the different time conditions. One-way ANOVA testing showed significance across the data, between the 0-hour control condition and all other conditions, and between the 12-hour control and 24-hour osmotic stress condition.

Cell Cycle Stage Analysis by FUCCI Fluorescence

The cell cycle stage of FUCCI RPE cells after osmotic stress was found by quantifying the percentage of cells in the G1 phase (red only), G1/S phase (both), and S/G2 phase (green only) for each condition. This was done to determine if cell synchronization had occurred. FUCCI RPE cells were given either osmotic media or regular media for either 12 or 24 hours. These conditions were run in duplicate. One set was fixed through centrifugation and formaldehyde and

then run through flow cytometry. Due to insufficient data collection resources and equipment, the flow cytometry was not able to be analyzed. The other set of cells was imaged using a 60x confocal microscope with fluorescence capabilities (Figure 7). The images were analyzed by tallying the number of cells fluorescing red-only, green-only, and both divided by the number of total cells in each image for each condition. An interleaved scatter plot with SEM error bars was created for the grouped data based on time conditions and cell color (Figure 8). The significance across the data and between pairs of conditions for each color was found using a two-way ANOVA test. The average percentage of red-only cells in an image was 0.5400 for the 24-hour control, 0.5389 for the 24-hour osmotic stress condition, 0.7210 for the 12-hour control, 0.6121 for the 12-hour osmotic stress condition, and 0.5964 for the 0-hour control. The average percentage of green-only cells in an image was 0.1369 for the 24-hour control, 0.07778 for the 24-hour osmotic stress condition, 0.05606 for the 12-hour control, 0.1197 for the 12-hour osmotic stress condition, and 0.1221 for the 0-hour control. Finally, the average percentage of both red and green cells in an image was 0.3231 for the 24-hour control, 0.3833 for the 24-hour osmotic stress condition, 0.2229 for the 12-hour control, 0.2682 for the 12-hour osmotic stress condition, and 0.2815 for the 0-hour control. No significance was found across the conditions for any of the cell colors.

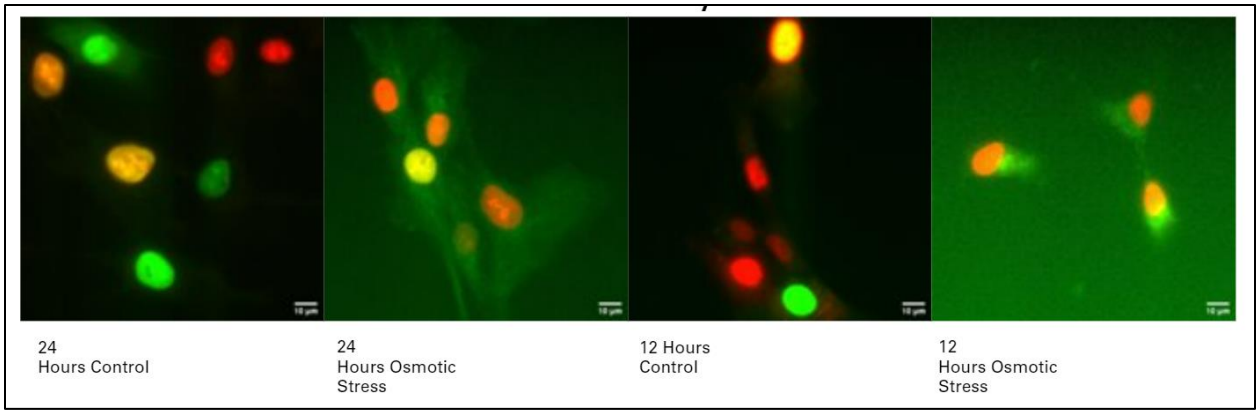

Figure 7. Images of fixed FUCCI cells with FITC (Green) and TRITC (red) fluorescence at different time conditions (L-R): 24-hour control, 24-hour osmotic stress, 12-hour control, 12-hour osmotic stress. Scale bars showing 10 µm are included. Yellow/orange cells indicated both red and green fluorescence.

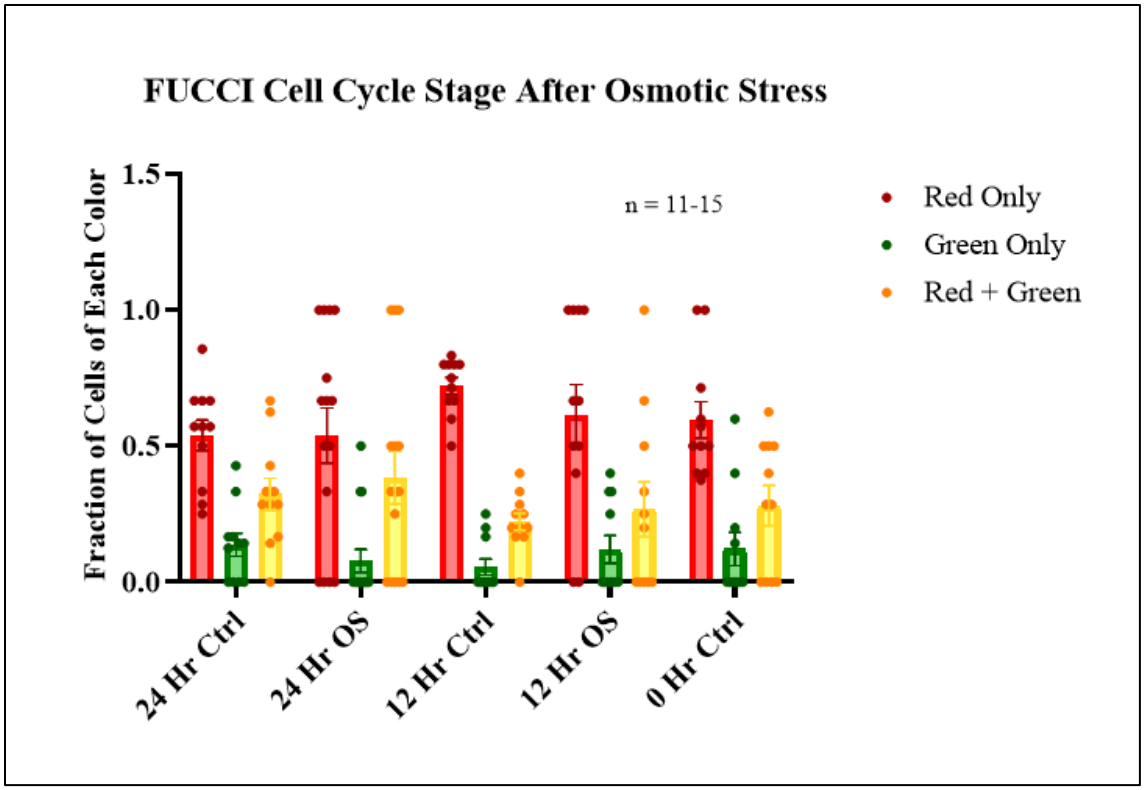

Figure 8. Interleaved scatter plot with SEM error bars the fraction of cells of each color across time conditions and color groups. Y-axis shows the fraction of cells of each color group within individual images taken. X-axis shows the different time conditions. Individual column bars represent one of the three color groupings (red only, green only, and both red and green) Two-way ANOVA testing showed no significance across the data or for any pairs of conditions.

Discussion

Cell circadian rhythms, though having such a large effect on bodily functions, are not well understood. Many new studies contradict previous understandings of the circadian rhythm cycle including its recently-discovered independence from light and ability to function even when individual elements, even BMAL1, are removed. One area of study still in its early stages of research is how the circadian system reacts to environmental stressors on a cellular level. The molecular mechanism of the circadian cycle involves many different moving parts and while it's known to be affected by signals from the environment, it is not always clear what elements are directly impacted. This study aimed to further explore the impacts of osmotic stress on the cell circadian rhythms in different cell lines to determine a correlation between caveolae deformation and BMAL1 localization as well as to determine whether osmotic stress induces cell cycle arrest across cell lines.

Serum starvation has long been used as a method to synchronize cells in the G1 growth stage before further experimentation. By synchronizing cells, an experiment tracking cell growth or metabolism can be run without any other determining factors. Despite the method's commonality, there is no consensus for the most effective length of serum starvation to synchronize cells. In order to most effectively synchronize the PC12 cells before osmotic stress, an experiment testing serum starvation time was run. A cell's growth cycle stage can be easily determined by measuring and comparing the area of the nucleus. If the cell stopped growth in the G1 stage, then the nucleus would be smaller than in the S stage where DNA replication occurs or in the following G2 stage.

As expected, all serum starvation conditions tested showed cell cycle arrest in the G1 stage with no significance between these conditions. To further examine each condition's effectivity, the population of viable cells was then qualitatively compared. Though the 24-hour serum starvation had the smallest mean nuclear area, it also had the smallest cell population and therefore would not be a good fit, especially for an experiment that intentionally stresses cells. This result made sense given that not many cells would be expected to survive 24 hours of prolonged serum starvation. Both the 8-hour and 16-hour conditions had similar mean nuclear areas and significance compared to the controls. As 16 hours of serum starvation produced more viable

cells than 8 hours did, this time was chosen for use in synchronizing cells for the BMAL1 localization experiment. One unexpected result was the lack of viable cells from the 12-hour serum starvation condition. This may have been due to an improper fixation procedure using diH_2O in place of PBS during fixation which may have further stressed the cells causing mass cell death and a build-up of debris. This experiment should be replicated with a more appropriate fixation procedure and all four time conditions (8 hours, 12 hours, 16 hours, and 24 hours). Additional experimentation should also be conducted to determine if serum starvation is consistent across cell lines.

During the cell circadian cycle, BMAL1 is primarily localized to the nucleus when dimerized with CLOCK and participating in E-box binding. BMAL1 can also bind cytoplasmic proteins to regulate mRNA translation outside of the TTFL. BMAL1 localization was used to track the impact of osmotic stress on the circadian rhythm cycle due to its key role in the TTFL and its predictable localization patterns when engaged in the TTFL. Previous research showed that osmotic stress causes caveolae deformation and stress granule accumulation in the cytoplasm in WKO cells. This study also found that clock gene expression was affected by osmotic stress. From this they hypothesized that caveolae deformation leads to a disruption in the TTFL through the resulting accumulation and binding with BMAL1 of stress granule proteins in the cytoplasm, inhibiting BMAL1/CLOCK dimerization.

PC12 cells were used to test osmotic stress impacts due to their low levels of caveolae, meaning they would not be expected to experience a disruption to the circadian cycle through BMAL1 localization to the cytoplasm when osmotically stressed assuming caveolae deformation is the cause of this effect. Localization of BMAL1 in the cytoplasm when it would otherwise be found in the nucleus indicates BMAL1 phosphorylation in the cytoplasm disrupting the cell circadian cycle. Controls consisting of regular media refeeding after serum starvation were used to track BMAL1 localization as it would occur in a normal circadian cycle. After serum starvation, when cells should be in the G1 phase, BMAL1 was found primarily in the nucleus. For all other conditions, BMAL1 intensity in the nucleus was significantly lower and increased over time, indicating a naturally longer circadian cycle for PC12 cells than WKO cells. Despite the change in BMAL1 localization, data did not indicate any impact from osmotic stress. This supports the hypothesis that caveolae deformation is a crucial element impacting the cell circadian rhythm

and BMAL1 localization. Further experimentation should be conducted to confirm the results in other cell lines not containing caveolae. Additionally, analysis of caveolae presence in PC12 cells would provide more insight into the observed results. Similarly, tracking upstream gene regulation after osmotic stress using PCR and sequencing would be useful to determine other affected genes and related pathways in PC12 cells and to confirm the results.

While osmotic stress may not have a consistent effect on all cell lines, it has been hypothesized that due to its impact on the circadian rhythm pathway, it may be useful as a means of cell synchronization through cell cycle arrest. Another cell line, FUCCI-modified RPE cells, which typically have caveolae, were used to test osmotic stress induced cell synchronization. Since RPE cells have caveolae they would be expected to react similarly to osmotic stress as WKO cells. The FUCCI system was used to determine cell cycle stage as FUCCI cells are genetically designed to fluoresce different colors at the G1 and S/G2 stages. Data analysis was conducted through qualitative observation of cell fluorescence in images. Another means of analyzing the impact of osmotic stress on the cell cycle would be to take nuclei area measurements as done with the serum starvation experiment. This would provide more objective quantitative data through which to confirm the results.

Though preliminary observations indicated that osmotic stress may have had an impact on cell growth, comparisons of the fractions of cells of each color for each condition showed no synchronization in osmotically stressed cells. This may be due to how osmotic stress impacts other pathways in the cell and how the circadian rhythm pathway interacts with cell metabolism. If the pathways directly causing cell growth are not disrupted or have other regulatory factors not related to the circadian rhythm system, then they may have been able to continue even under osmotic stress. Further studies using DNA sequencing to determine the impact of osmotic stress on cell growth mechanisms should be conducted to monitor what genes and what pathways are being affected apart from the circadian system. Additionally, BMAL1 tracking and caveolae analysis could be used to confirm that RPE cells behave the same as WKO cells when subjected to osmotic stress. There is still so much unknown about how cellular pathways are affected by osmotic stress and the interconnections between them. Further studies are necessary to better understand these pathways and to confirm their roles and mechanisms across cell lines.

Conclusion

The experiments performed on PC12 cells and FUCCI RPE cells demonstrate that cell line plays an important role in osmotic stress response and that cells not containing caveolae are less likely to undergo BMAL1 phosphorylation when subjected to 12 hours of osmotic stress compared to cells with caveolae. The results of the serum starvation experiment indicate that prolonged serum starvation will cause cell cycle arrest in the G1 phase across multiple lengths of application, though longer lengths of time may cause high levels of cell death. Further studies are necessary to confirm these results. BMAL1 localization in PC12 cells after osmotic stress supported previous research connecting caveolae deformation to disruption of the circadian rhythm. Future research should be done to confirm these results in other cell lines and through gene sequencing. In FUCCI RPE cells, osmotic stress did not induce cell cycle arrest and data indicated that osmotic stress alone is not an effective method of cell synchronization. Osmotic stress may still have an impact on cell growth and may have other uses in cell cycle research yet to be discovered. There is still much to learn about the impacts of osmotic stress on cellular pathways and their molecular mechanisms. This area of study will provide insight into issues of homeostasis and circadian rhythm regulation to be used in future research and potential medical treatments of circadian rhythm disorders.

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